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Blood Substitutes

Edited by Robert Winslow

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Preface

The precursor of this book was *Hemoglobin-based Red Cell Substitutes*, published by the Johns Hopkins Press in 1992 (Winslow, 1992). The initial idea for the current book was to produce an updated edition, but somewhat less austere and more accessible to a wider readership. As we worked on this idea it became less and less appealing, partly because the field has advanced so rapidly and so far since 1992. Furthermore, I became less and less confident that I would be able to do justice to the vast amount of new research that has been done since that time. Therefore it was decided that this book would be multi-authored.

The book has several aims. First, it is intended to be comprehensive. Invitations to contribute were accepted by all but a few potential authors, and, except for a few hard cases, the authors have been extraordinarily prompt in accomplishing their contributions. Second, the book aims not to be a collection of disconnected essays, as so often happens in symposium proceedings; rather authors were asked to cover their field of specialization with background for the non-specialized reader before delving into the details of their own research. Third, the book aims for a degree of cohesiveness not usually found in multi-authored books. We have tried hard to ensure uniformity of usage and standardization of abbreviations. During the writing and editing stages, I frequently asked authors to resolve conflicts with other chapters and to document statements that seemed to me to be matters of opinion. Fourth, we have tried to make the book accessible to the general reader. To do this, I have written a short abstract of each chapter; these abstracts are clearly identified as editor's comments.

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Margaret Macdonald and Victoria Lebedeva at Elsevier have been totally supportive at each stage of the project. I shall miss our monthly meetings in London with tea and scones. At Sangart, Pam Boltz has skillfully managed all of the daily chores of interacting with authors about details, prodding them to keep to deadlines and managing the profusion of computer files that accumulate as revisions are collected.

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Abbreviations

2,3-DPG	2,3-diphosphoglycerate, an allosteric effector of hemoglobin O_2 binding
2-IT	2-iminothiolane (Traut's reagent)
3,4 DHBA	3,4 dihydroxybenzoate
$\alpha\alpha$-Hb	Hemoglobin crosslinked between α chains at Lys92 with bis-3,5(dibromo)salicylic acid (DBBF)
AABB	American Association of Blood Banks
A-ANH	Augmented acute normovolemic hemodilution
AAR	Abdominal aortic reconstruction
ABC	America's Blood Centers
ABO	Red blood cell surface antigens
ADP	Adenosine diphosphate
AE	Adverse event
AHD	Acute preoperative hemodilution
AHSP	Alpha hemoglobin stabilizing protein
ALT	Alanine aminotransferase
ANH	Acute normovolemic hemodilution
aPTT	Activated partial thromboplastin time
AR	Acetated Ringer's solution USP
ARC	American Red Cross
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
ATLS	Advanced trauma life support
ATP	Adenosine triphosphate, the main substrate for energy metabolism
AVM	Arteriovenous malformations
BAEC	Bovine aortic endothelial cells
BBB	Blood – brain barrier
BLA	Biological license application
BSE	Bovine spongiform encephalitis
BVDV	Bovine diarrhea virus
BvHb	Bovine hemoglobin
C(a – v)O_2	Arterial – mixed venous O_2 content difference
C3	A component of complement
C5a	A component of complement
CABG	Coronary artery bypass graft
cAMP	Cyclic adenosine monophosphate
CBER	Center for Biologics Evaluation and Research (a component of the FDA)
CCLI	Chronic critical limb ischemia
CHO	Chinese hamster ovary cells
CI	Cardiac index (cardiac output divided by bodyweight)
CJD	Creutzfeldt – Jakob disease
CK-MB	Creatinine kinase, myocardial isoenzyme
CNmetHb	Cyanomet hemoglobin
CNS	Central nervous system
CO	Cardiac output
COP	Colloid osmotic pressure (oncotic pressure)
CPB	Cardiopulmonary bypass
D5W	5% dextrose (glucose) in water, an intravenous solution
Da	Dalton, a unit of molecular weight
DAB	Diaminobenzoate
DBBF	bis-3,5(dibromo)salicylic acid, a hemoglobin crosslinker
DBBF-Hb	Same as $\alpha\alpha$-Hemaglobin
DBBF-HvHb	Bovine hemoglobin crosslinked with DBBF
DCLHb	Diaspirin crosslinked hemoglobin Baxter's preparation of $\alpha\alpha$-Hb
DCS	Decompression sickness
DECA	Sebacyl crosslinked hemoglobin
DHA	Dehydroascorbic acid
DHBA	2,3- and 2,5-dihydroxybenzoic acids
DIC	Disseminated intravascular coagulation

DMC	Degranulating mast cells
DMF	Drug master file
DMPG	Dimyristoyl-phosphatidyl glycerol
DNA	Deoxyribonucleic acid
DO_2	Oxygen delivery: cardiac output × arterial O_2 content
dP/dt	The maximum positive slope of the arterial pressure curve
DPPC	Dipalmitoylphosphatidylcholine
DPPG	Dipalmitoyl-phospatidyl glycerol
DSMB	Data Safety Monitoring Board
DSPC	Distearoylphosphatidylcholine
DxHb	Dextran-linked hemoglobin
EBL	Estimated blood loss
eBTr	Embryonic bovine trachea cells
EBV	Exchangeable blood volume (also estimated blood volume)
EC50	The effective concentration of a solution that kills 50 per cent of neurons in culture
ECG	Electrocardiogram
ECMO	Extracorporeal membrane oxygenation
EDRF	Endothelium derived relaxing factor
EDTA	Ethylene diamine tetraacetic acid
Ees	End systolic elasticity
EMEA	European Medicines Agency
eNOS	Endothelium-derived nitric oxide synthase
EPO	Erythropoietin
EPR	Electron paramagnetic resonance
EVA	Ethyl vinyl acetate
EYP	Egg-yolk phospholipid
FCD	Functional capillary density
FDA	US Food and Drug Administration
FDMA	Food and Drug Modernization Act
FiO_2	Fraction of inspired oxygen
FOI	Swedish Defense Establishment
GCP	Good clinical practices
GFR	Glomerular filtration rate
GLP	Good laboratory practices
GMP	Good manufacturing practices
GPC	Gel permeation chromatography
GSH	Glutathione
H1S	Hemopure 1 solution (Biopure)
H1S-2	Similar to H1S but with reduced unpolymerized hemoglobin
HAS	Human serum albumin
HAV	Hepatitis A virus
Hb	Hemoglobin
HbA	Hemoglobin A, the main hemoglobin of the human red blood cell
HbA_0	HbA purified by chromatography
HBOC	Hemoglobin-based oxygen carrier
HBOC-201	A formulation of glutaraldehyde-polymerized bovine hemoglobin produced by Biopure Corp.
HBOC-301	A formulation of glutaraldehyde-polymerized bovine hemoglobin produced by Biopure Corp. for veterinary use (Oxyglobin™)
Hb-PEGP5K2	Hemoglobin modified by the attachment of two strands of PEG 5 kDa
Hb-PEGP5K6	Hemoglobin conjugated to six strands of PEG 5000 kDa
Hb-PEGP10K2	Hemoglobin modified by the attachment of 2 strands of PEG 10 kDa
HbS	Sickle cell hemoglobin
HBV	Hepatitis B virus
Hct	Hematocrit, the percentage volume of blood that is red blood cells
HCV	Hepatitis C virus
HES	Hydroxyethyl starch
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus, the causative agent of AIDS
HNLS	Hyperinflated non-collapsible lung syndrome
HO-1	Heme oxygenase 1
HO-2	Heme oxygenase 2
HO-3	Heme oxygenase 3
HS	Hypertonic saline
HSD	Hypertonic saline dextran
HSHb	Hypertonic saline hemoglobin
HSP70	Heat shock protein 70

HTLV	Human T lymphotropic cell virus
HUVEC	Human umbilical vein endothelial cells
IAD	Intraoperative autologous blood donation
ICH	Intracerebral hemorrhage
ICP	Intracranial pressure
ICU	Intensive care unit
IHP	Inositol hexaphosphate
IL-5	Interleukin-5
IL-6	Interleukin 6, an inflammatory cytokine
IND	Investigational New Drug application to the FDA
IRB	Institutional Review Board
ISS	Injury severity score
JCAHO	Joint Commission for Accreditation of Healthcare Organizations
kDa	kiloDalton, 1000 Daltons
LAD	Left anterior descending coronary artery
LAIR	Letterman Army Institute of Research
LDCR	Late diastolic coronary resistance
LDH	Lactate dehydrogenase
LEH	Liposome-encapsulated hemoglobin
L-NAME	L-N^G-nitro-arginine methyl ester
LPS	Lipopolysaccharide (endotoxin)
LR	Lactated Ringer's solution USP
MalPEG-Hb	Hemoglobin modified by attachment of maleimide-activated PEG 5000
MAP	Mean arterial pressure
Mb	Myoglobin
MCA	Middle cerebral artery
MCP-1	Monocyte chemoattractant protein-1
MMS	Monocyte-macrophage system (reticuloendothelial system)
MOF	Multiorgan failure
MP4	MalPEG-Hb formulated at 4.2 g/dl in lactated Ringer's solution
MPAP	Mean pulmonary artery pressure
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MVD	Maximum valid dilution
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP
NAG	N-acetyl-α-D-glucosaminidase
NAT	Nucleic acid amplification test
NDA	New Drug Application
NEM	N-ethyl maleimide
NFPLP	2-Nor-2-formylpyridoxal 5′-phosphate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NRC	Neo red cells
OEC	Oxygen equilibrium curve
OER	Oxygen extraction ratio
o-R-Poly-Hb	Hemoglobin polymerized with o-Raffinose, a Hemosol product
*P*50	The PO_2 at which hemoglobin is half-saturated with oxygen
$PaCO_2$	PCO_2 of arterial blood
PAD	Perioperative autologous donation of blood
PaO_2	PO_2 of arterial blood
PAOD	Peripheral arterial occlusive disease
PCO_2	Partial pressure of CO_2
P_{DH}	Hemoglobin permeability coefficient
PEG	Polyethylene glycol
PEG-BvHb	Bovine hemoglobin modified by surface conjugation to PEG
PEG-Hb	Bovine hemoglobin modified by surface conjugation to polyethylene glycol (PEG), a product of Enzon, Inc.
PEG-PE	Phosphatidylethanolamines
PET	Positron emission tomography
PFC	Perfluorocarbon
PFD	Perfluorodecalin
PFMCP	Perfluoro-N-4-(methylcyclohexyl)-piperidine
PFOB	Perfluorooctyl bromide (Perflubron)
PFTMCH	Perfluorotetramethylcyclohexane
PFTPA	Perfluorotripropylamine
PGI_2	Prostacyclin I_2
PHP	Pyridoxalated hemoglobin polyoxyethylene

PLA	d,l-polylactic acid
PLP	Pyridoxal-5′-phosphate, an allosteric effect of hemoglobin O_2 binding
PMB	(or PCMB) Perchloromercurobenzoate
PMN	Polymorphonuclear cells (white cells, leukocytes)
PO_2	Partial pressure of oxygen
POD	Postoperative day
PolyBvHb	Polymerized bovine hemoglobin
PolyHb	Polymerized hemoglobin
PolyHb–SOD	Polymerized hemoglobin conjugated to superoxide dysmutase
PolyHeme	Glutaraldehyde-polymerized human hemoglobin (Northfield)
PPTA	Plasma Protein Therapeutics Association
PRBC	Packed red blood cells
PRV	Pseudorabies virus
PS-ODN	C-10 phosphorothioate oligodeoxynucleotide
PT	Prothrombin time
PTCA	Percutaneous transluminal coronary angioplasty
PTFE	Polytetrafluoroethylene
PtO_2(M)	Muscle PO_2
PtO_2(R)	Renal tissue PO_2
PTP	Post-transfusion purpura
PV	Plasma volume
$PvCO_2$	Mixed venous PCO_2
PvO_2	Mixed venous PO_2
PVR	Pulmonary vascular resistance
Q	Abbreviation for flow (most often cardiac output), used in equations
QLS	Quasi-elastic light scattering
RA	Acetated Ringer's solution USP
RBC	Red blood cell
RES	Reticuloendothelial system
R_G	Radius of gyration
rHb	Recombinant hemoglobin
RhoD	Rh blood group
rHSA	Recombinant human serum albumin
RLM	Rat liver mitochondria
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
ROSC	Return of spontaneous circulation
SAAP	Selective aortic perfusion
SAE	Severe adverse event
SAH	Subarachnoid hemorrhage
SaO_2	Arterial hemoglobin saturation
SBP	Systolic blood pressure
SCD	Sickle cell disease – one of several genotypes that produce sickling of RBC when deoxygenated
SC-PEG	PEG-succinimidyl carbonate
SFH	Stroma-free hemoglobin
SIRS	Systemic inflammatory response syndrome
SMA	Superior mesenteric artery
SNAP	S-nitroso-N-acetylpenicillamine
SO_2	Hemoglobin saturation
STP	Sodium triphosphate
SvO_2	Mixed venous hemoglobin saturation
SVR	Systemic vascular resistance
TA-GvHD	Transfusion-related graft-versus-host disease
TBI	Traumatic brain injury
TEE	Transesophageal echocardiography
TEM	Transmission electron micrograph
TFF	Tangential flow filtration
TNF	Tumor necrosis factor, an inflammatory cytokine
TPA	Tissue plasminogen activator
TRALI	Transfusion-related acute lung injury
TRIM	Transfusion-associated immunomodulation
UV	Ultraviolet
vCJD	Variant CJD, a form of CJD that has infected some humans
VEGF	Vascular endothelial growth factor
VO_2	Oxygen consumption: cardiac output $\times$ (a – v) O_2 context difference
VSV	Vesicular stomatitis virus
ZL-BvHb	'Zero linked' bovine hemoglobin

Introduction

In his 1937 review on blood substitutes, Amberson wrote:

> *The blood of vertebrates is the most complicated fluid to be found in the world of living organisms. Compounded of a dozen essential ingredients, sustaining a multiplicity of activities, the fluid pathway for a variety of chemical and hormonal integrations of function, the source of food and oxygen for every tissue, it defies laboratory synthesis. At the very beginning we must recognize that there is no complete substitute for blood. Yet biologists and physiologists, no less than clinicians, are so frequently confronted with situations where normal blood cannot be obtained, or where the problem at issue can only be solved by a simplification of conditions, that a substitute for blood has become one of the most pressing needs of the experimental laboratory.*
>
> (Amberson, 1937)

More than sixty years later there is still no substitute for blood to be used for transfusion, in spite of substantial efforts to produce one. This book will review some developments that relate to red cell substitutes and will present some of the forces that have driven research in the field. At the outset, the obvious may be stated: if it were a simple problem, it would have been solved long ago.

The US Army, which contributed so much to the blood-banking system and to the development of human serum albumin as a therapeutic agent, has long recognized the impact that a universal oxygen-carrying resuscitation fluid would have in the care of trauma victims. Wartime requirements placed the military at the center of research and development efforts; the military is perceived as a certain market for oxygen-carrying solutions. Research has been carried out in both military and civilian laboratories for more than thirty years since it was discovered that hemoglobin could be modified chemically to improve its usefulness as a cell-free oxygen carrier.

Efforts to develop products for non-military uses intensified in the 1980s for several reasons. First, the supply of blood for transfusion in the United States was diminishing, partly because of more efficient usage of banked blood and partly because of dropping rates of donation. The acquired immune-deficiency syndrome (AIDS) epidemic made both physicians and patients aware that there is an inherent risk in receiving banked blood. Efforts to control the human immunodeficiency virus (HIV) have drawn attention to other pathogenic viruses that can potentially contaminate blood. Finally, the perception of huge profits stimulated many commercial programs to produce 'artificial blood' in the 1980s.

With all this interest, scientific focus and invested capital, why has the promise of an artificial oxygen carrier not been fulfilled? Frequently such failure can be attributed to the fact that related fields are also relatively undeveloped, and fundamental insights into basic biologic mechanisms are lacking (Comroe, 1976). Such is the nature of the red cell substitute problem.

Ever since William Harvey described blood circulation in 1628, a red cell substitute has seemed nearly at hand. Nevertheless, in 2005 only one product is in Phase III clinical trials. It is the intention of this book to review the efforts that have been made up to now, and to try to bring forth conclusions from this massive amount of work that might point the way to a successful future. Although a commercial product is not in hand, it is an exciting story of investigation into fundamental mechanisms of oxygen transport to tissue, of specific toxicities and of an exploration of the huge potential for new products that could supply oxygen to tissue at risk – such as in heart attacks, stroke and trauma.

This book brings together in one source the current research and state of the art development of the leading workers in the field. It is a testament to the enthusiasm and dedication of

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these authors that with almost no exception, each of them agreed to participate in this project and to share their most recent findings, thoughts and opinions. It is hoped that the book will provide a ready source of information that can be accessed by new scientists, clinicians and related persons who might be interested in joining the search for new therapeutic agents that significantly impact so many patients.

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SECTION

1

Background

The background section reviews the long history of the quest for 'artificial blood' – a goal that has still not been achieved. Some of the reasons for this frustration are reviewed and put into the context of historical development of related fields of science and medicine. While research has been evolving, the safety of blood transfusions has increased dramatically over the past two decades, resulting in a 'moving target' for those working in the field. Regulatory agencies, such as the US FDA, have worked diligently, within legal guidelines, to define requirements for commercial approval. These guidelines provide a 'roadmap' for development.

Chapter 1

Historical Background

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INTRODUCTION

The histories of blood transfusion and red cell substitutes are complementary: without a need for transfusions, there would be no need for alternatives. More than 300 years passed between the first description of blood circulation in the body and the implementation of routine transfusion in medicine. It is not surprising that a little more time is needed to develop safe, practical red cell substitutes (see Table 1.1).

BLOOD CIRCULATION AND TRANSFUSION

The lore of blood transfusion is rooted in antiquity. *Leviticus* 7:26, which forbids the eating of blood, is the basis today for the refusal by certain religious groups to receive transfusions. However, the ancient Egyptians used 'blood baths' as a restorative, and Romans apparently rushed into the arenas to drink the blood of dying gladiators. However, no transfusion could occur until the circulation of the blood was understood.

Although Ibn Nafis described the pulmonary circulation in the thirteenth century (Comroe, 1976), William Harvey described his theory of the circulation of the blood independently in 1616 and published his views in 1628 for the first time in the Western literature (Harvey, 1653). Harvey had been trained in Padua to acquire knowledge by direct observation, a method he used to great advantage in England (Dormandy, 1978). His magnificent contribution to science was the key that opened the door to modern studies of blood transfusion, and almost immediately after its publication, efforts in the quest for red cell substitutes began.

The first successful blood transfusion from one animal to another was probably performed in 1665 by Richard Lower and Edmund King (Hollingsworth, 1928). Such early efforts used the quills of bird feathers to puncture arteries or veins, and blood was collected in animal bladders. Not surprisingly, there were many failures! It is of interest that Lower's experiments were directed at perfecting the technique of injection with various solutions such as wines and beers. In the course of his experiments he noted that these solutions mixed freely with blood except when mixed *ex vivo*. Therefore, to test their miscibility, he injected them intravenously (Hollingsworth, 1928).

Lower's success captured the imagination of the Royal Society of London, and he received widespread attention for it. There was rampant speculation on the question of whether a dog would grow wool, hoofs and horns after transfusion with sheep's blood. Samuel Pepys speculated that the new practice of transfusion 'did give rise to many pretty wishes, as of the blood

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Table 1.1 The history of oxygen therapeutics (blood substitutes)

Year	Event
1628	Circulation of the blood (Harvey)
1656	Wine, scammony, opium, blood (Wren)
1665	First animal transfusion (Lower)
1667	First human transfusion (Denis), death, moratorium
1818	Renewed interest in transfusion (Blundell)
1835	Defibrination of blood (Bischoff)
1863	Gum-saline (Ludwig)
1867	Bacteria, fungi, asepsis (Pasteur; Lister)
1871	Plasma and serum
1878	Milk, cholera epidemic (Thomas; Jennings; Hodder; Bovell)
1900	Red cell antigens (Landsteiner)
1916	Hemoglobin infusions in humans (Sellards and Minot)
1937	Amberson's review
1941–45	Albumin, hemoglobin solutions
1949	Amberson's report of hemoglobin infusions in humans
1957	Encapsulated hemoglobin (Chang)
1966	'Bloodless' mouse (Clark and Gollan)
1967	Stroma-free hemoglobin (Rabiner *et al.*)
1968	Exchange transfusion with PFC (Geyer *et al.*)
1968	Hemoglobin dimerization demonstrated (Bunn and Jandl)
1972	Modification of hemoglobin to reduce oxygen affinity (Benesch *et al.*)
1973	Glutaraldehyde polymerization of hemoglobin (Payne)
1976	'Polyhemoglobin' (Bonhard)
1978	Human safety trial, unmodified hemoglobin (Savitsky)
1989	Human trials with modified hemoglobins (Moss)
1992	US Army abandons $\alpha\alpha$-hemoglobin (Hess)
1998	Baxter abandons $\alpha\alpha$-hemoglobin
2004	Alliance Pharmaceutical abandons PFC emulsion trials

of a Quaker to be let into an Archbishop and such like' (Nicolson, 1965). It was also proposed that the phlegmatic personality could be corrected by transfusion with blood from a choleric, and even that marital discord could be settled by reciprocal transfusion of husband and wife.

An important observation was also made by Pepys:

> Above all I was pleased to see the person who had his blood taken out. He speaks well, and did this day give the Society a relation thereof in Latin, saying that he finds himself much better since, and as a new man.

Apparently there was some concern that the act of blood donation was not safe.

Although one romantic story describes the collection of blood from three boys to transfuse into Pope Innocent VIII in 1492 (Lindeboom, 1954), the first human transfusions were actually performed in France. Perhaps the first successful one was the work of Jean Baptiste Denis, the physician to Louis XIV, in June 1667. A young boy who suffered from an obscure illness and had been treated by venesection to the point of exhaustion received a small amount of lamb's blood and made a remarkable recovery. In the wake of this success, Denis transfused additional patients until one, upon receipt of a third transfusion, died, apparently because of an incompatibility reaction. Denis was charged with murder but was eventually exonerated. This experience, and the publicity it stirred, led to a moratorium on transfusion practice in France, England and Italy, and research abated.

The search for a red cell substitute paralleled the search for safe blood transfusion. In retrospect, both quests were doomed because of the need for basic advances in related fields. Initially, however, it was thought that transfusion of blood was completely unsafe and that only alternatives could be used. Therefore the eventual need for a substitute decreased when safe transfusion practices became available.

Interest in transfusion was revived by the obstetrician James Blundell in 1818. Blundell, faced with uncontrolled fatal puerperal hemorrhage, directed his scientific efforts to the totally neglected operation of blood transfusion. He found the field dominated by antiquated ideas that the blood was 'alive'. The famous surgeon John Hunter wrote in 1817:

> One of the great proofs that the blood possesses life depends upon the circumstances affecting its coagulation. If the blood had not the living principle it would be in respect to the body as an extraneous substance.
>
> (Jones and Mackmul, 1928)

Blundell devoted himself to perfecting the techniques and devices for the safe and efficient collection of blood for transfusion. He transfused a total of ten patients. Of these, two were already

dead, and a third was dying of cancer. His five successes must have been very impressive at the time, and he is properly credited with reviving the use of transfusion and with putting it on a new scientific foundation. Among Blundell's contributions were the concepts that species lines should not be crossed, that it was not necessary to replace all the lost blood, and that small amounts of air injected into the circulation were not necessarily fatal. In addition, he invented a water-jacketed collection funnel and a donor chair similar to those used in modern blood banks (Jones and Mackmul, 1928).

When Blundell began his work, the chief potential for blood transfusion was thought to be resurrection. He believed that 'death from bleeding (like that of hanging or submersion) may also for a time be apparent … it is not impossible that transfusion may be of service within a given time even after breathing has stopped' (Blundell, 1824). However, he injected 16 ounces of blood into a woman who had been dead for about 6 minutes, and must have convinced himself that resurrection was an unrealistic goal!

Blundell apparently feared that untoward results might give what he believed to be the erroneous impression that transfusion should be abandoned, and he suggested that adverse cases should not be reported until 'a complete body of evidence upon the subject be obtained' (Jones and Mackmul, 1928). Of course this proposition was rejected, but his suggestion is very much parallel to today's trend toward not publishing results of experiments with red cell substitutes in the open literature for fear of discouraging progress.

Blundell became embroiled in controversy over his surgical techniques and his revival of the practice of transfusion. He left medical practice after performing only ten transfusions, and enjoyed a long retirement. Although there were many attempts to continue Blundell's work, results were sporadic and difficult to understand. Progress was hindered by the lack of understanding of hemolysis, coagulation and infection. Isolated successes were reported in the literature, but no consistent results could be obtained until these problems were understood.

Coagulation was the first problem to be solved. Bischoff (1835) described the defibrination of blood. By 1875 approximately 347 cases of transfusion were reported in the literature, 129 of which were with animal blood. Apparently only about half of them were successful, and the procedure was reserved for extreme cases, particularly severe hemorrhage (Landois, 1875). After Louis Pasteur had demonstrated that fungi and bacteria caused putrefaction, Joseph Lister introduced aseptic techniques in 1867. Thus the two major problems of coagulation and infection were solved over a period of about 30 years.

The remaining problems were not to yield for many more years, and these stimulated a renewed search for a red cell substitute. A brief flurry of interest in transfusion with lamb's blood followed, but a growing awareness of incompatibility prevented its widespread use. In addition, in the period around 1875 physiologic saline was introduced, and its safety and efficacy in cases of hemorrhage were demonstrated easily.

The greatest single advance in the use of blood transfusions came in 1900 with the demonstration by Karl Landsteiner (1901) of the presence of isoagglutinating and isoagglutinable substances in the blood. Such substances were shown to be responsible for incompatibility reactions and hemolysis. Many blood groups were described subsequently. The addition of anticoagulants solved another major problem when Hustin (1914) of Belgium reported his experiments using sodium citrate and glucose in the prevention of coagulation. By 1921 the three dangers of heterologous transfusion – incompatibility, infection and coagulation – were largely controlled. Thus, the need for a red cell substitute decreased again.

WORLD WAR II

The Cook County Hospital in Chicago is often credited with establishing the first modern blood bank in 1937 (Diamond, 1965), although the Rowan Memorial Hospital in Salisbury, North Carolina established a plasma storage facility in 1935 (Schmidt, 2000). At the time, efforts were directed at the collection and storage of both liquid and dried plasma in anticipation of wartime use, after a plea from the British Red Cross in 1940 that the American National Red Cross should send plasma for use there.

There was no organized system for the collection, processing and distribution of blood and blood products at the time of the American entry into World War II. Thus the war had a tremendous effect on the blood-banking system and on the use of blood and blood products in the United States. In England in 1943 Loutit and Mollison developed a mixture of acid citrate and dextrose,

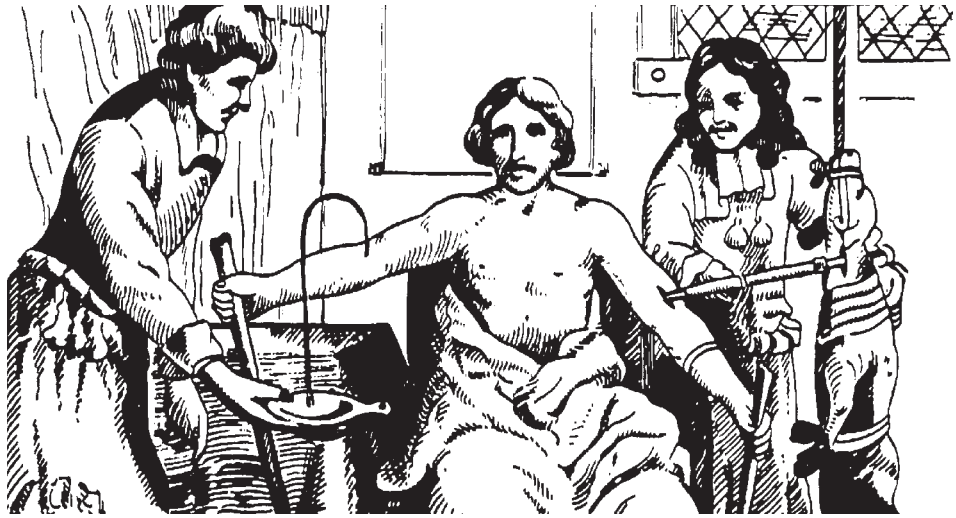

Figure 1.1 Blood transfusion from animal to man in 1672 (from Kilduffe and DeBakey, 1943, with permission).

which permitted the storage of blood for 21 days with a 70 per cent viability of cells. This achievement led directly to the widespread use of blood transfusion on the battlefield, and a remarkable lowering of morbidity and mortality.

The American Red Cross collected 13 million units of blood between 1941 and 1945, mainly for processing into dried plasma and, later, albumin. The first Allied shipment of blood to the European theater was in August 1944. These units used acid citrate dextrose, and were stored for up to 38 days. In all, 380 000 units were used in Europe and 180 000 in the Pacific theater.

After the war, the many returning physicians and surgeons who had come to rely on these products on the battlefield found they required blood banks and stored blood for their civilian practices. This led to rapid expansion of the blood bank system in the United States. In 1947 the American Red Cross blood banks were established, and by 1963 there were 56 of them. In 1948 the American Association of Blood Banks was formed, and the first Red Cross regional blood center was opened in Rochester, New York. By 1976, ten million units of blood were being collected per year.

FIRST 'BLOOD SUBSTITUTES'

The idea of red cell substitutes is not new. In Ovid's *Metamorphosis*, the witch Medea restored Jason's aged father, Aeson, by slitting his throat to let out old blood and replacing it with a magic brew she had concocted (Diamond, 1980). Sir Christopher Wren was one of the first to apply the new knowledge about the circulation to blood substitutes. In 1656 he infused ale, wine, scammony (a gummy exudate of the plant *Convolvulus scammonia*, a folk-medicine cathartic) and opium into dogs to study their effects. From these efforts he conceived the idea of transfusing blood from one animal to another as, he claimed, had been suggested to him by the story of Medea and Jason. However, Wren apparently did no more than suggest the possibility of transfusions to Lower, who actually carried out the experiments (Hollingsworth, 1928; Figure 1.1). Wren spent the rest of his long life working in the fields of astronomy and architecture rather than medicine, and he never returned to transfusions or red cell substitutes.

Milk

Milk was one of the first materials to be used as a red cell substitute (Thomas, 1878; Jennings, 1885; Ringer, 1885; Guthrie and Pike, 1907; Oberman, 1969). Edward Hodder used milk in cases of Asiatic cholera in 1854, and with Thomas he suggested that milk could regenerate white blood cells (Thomas, 1878). Two patients were given twelve ounces (or more) of cow's milk and did well, but two others died. In all, Thomas reported twelve cases and concluded that the injection of milk into the circulation in place of blood was a

perfectly feasible, safe and legitimate procedure. These results must have been very exciting, for according to John Brinton (1878): 'this new operation will, in a few years, have entirely superseded the transfusion of blood, which latter operation is even now being rejected as at once dangerous and unavailing in many parts of the country.'

There were several subsequent reports, and milk was shown to support function in isolated, perfused hearts from a variety of mammals (Guthrie and Pike, 1907); however, the transfusion of milk never gained widespread favor and soon disappeared from the literature.

Normal saline

In the laboratory, the search for a red cell substitute was directed at understanding the physiologic role of blood and its many components rather than at development of clinical applications. *Salzfrosche* were frogs whose blood was completely washed out and replaced with a pure sodium chloride solution. They survived for some hours. 'Urea-frogs' and 'sugar-frogs' lived longer, but if a small amount of red cells remained they could survive indefinitely. However, frogs are simple animals, and a frog's nervous system can be kept alive for some time without any circulation at all.

Ringer's solution

In 1883, Sydney Ringer discovered that the excised ventricle of the frog would beat for some hours if supplied with an aqueous solution of sodium, potassium and calcium salts. He found that the concentration of potassium and calcium was critical, whereas the amounts of the anions had little effect on the frog heart. The composition of 'Ringer's' solution (Table 1.2) was shown many years later to be very close to that of frog plasma. Probably the most popular crystalloid (salt) solution for intravenous use in humans is Ringer's lactate, in which lactate is added to Ringer's solution. The lactate is gradually converted to sodium bicarbonate within the body so that an uncompensated alkalosis is prevented (Hartmann and Senn, 1932). However, these 'crystalloid' solutions cannot support life without red cells; saline passes rather quickly into the tissue spaces of various organs (Miller and Poindexter, 1932), especially the liver (Lamson *et al.*, 1945).

Table 1.2 The composition of Ringer's solution

	Ringer's solution		Frog plasma
	(g/100 ml)	(mEq/l)	(mEq/l)
NaCl	0.6	102	104
KCl	0.0075	1.0	2.5
$CaCl_2$	0.01	1.8	1.0
$NaHCO_3$	0.01	1.2	25.4

Gum saline

Gum is a galactosidogluconic acid whose molecular mass is approximately 1500 Daltons. First used by Karl Ludwig in kidney perfusion experiments, gum-saline enjoyed great popularity as a plasma expander from the end of World War I onward. However, the aggregation state of gum depends on concentration, pH, salts and temperature; thus, its colloid osmotic pressure and viscosity are quite variable. Conditions under which the viscosity would be the same as that of whole blood were identified by Bayliss (1920).

In early animal studies, gum was found to coat the surfaces of all blood cells and to promote coagulation. The use of gum-saline became popular in World War I, but it was soon proved not to be efficacious in hemorrhagic shock if the hematocrit was less than 25 per cent. In the postwar period, Penfield (1919) showed that gum-saline was less effective than saline alone in treating hemorrhagic shock, but it was useful in stabilizing the blood volume temporarily (Henderson and Haggard, 1922). Although throughout the 1920s many reports of anaphylaxis and other untoward reactions appeared, Amberson (1937) claimed that when properly purified, gum-saline was safe for human use. Pharmacologic studies in the 1930s (Amberson, 1937) showed that gum was deposited in the liver and spleen and could remain there for many years. Its half-life in the circulation was about 30 hours, and anaphylaxis occurred occasionally. Success with gum-saline became common in the 1930s, but by that time the availability of plasma was such that the need for gum-saline decreased.

BLOOD PLASMA, SERUM AND ALBUMIN

The terms *plasma* and *serum* are frequently confused. Plasma refers to the liquid that suspends the red cells within the body. Serum is that liquid,

removed from the body, from which the coagulum has been extracted. This is a very important distinction, because serum contains no coagulation factors and is severely depleted of platelets.

As early as 1871 it was noted that a frog's heart could be maintained by perfusion with sheep and rabbit serum (Bowditch, 1871) and that this solution was superior to 0.6% sodium chloride (Kronecker and Stirling, 1875). Throughout ensuing years it was recognized that serum exerts a colloid osmotic pressure, contains bicarbonate, and may ensure capillary integrity. Ringer (1885), after dismissing a physiologic role for plasma lipids, eventually agreed that albumin added to a balanced salt solution was superior to the salt solution alone in maintaining the frog's heart.

Claude Bernard recognized that colloids (molecules such as proteins that do not cross biological membranes, or only slowly) were important in maintaining water balance, but Ernest Starling (1896) showed clearly that crystalloids (diffusing molecules such as salts) pass through biological membranes easily, whereas colloids do not. Thus, solutions of colloid exert an 'oncotic pressure' as water diffuses from the interstitium into vessels, drawn by the imbalance in colloids. In a classic paper, Gilbert Adair (1925) described in detail the measurement of the colloid osmotic pressure.

In the first half of the present century, much work was devoted to the study of plasma and serum as blood substitutes. One of the problems in this field was the recognition of toxic substances (Moldovan, 1910). Reports were published of intravascular coagulation and 'vasotonins' that appeared mysteriously after the infusion of serum or plasma. Some workers suggested that this activity could be reduced by heating the serum or by filtering it before use, and some suggested that platelets were to blame. Insulin was implicated by some, and adenosine triphosphate (ATP) by others. A major advance in understanding these problems came when the red cell surface antigens were elucidated because the use of serum from donors of blood group AB reduced the vasoconstrictor activity markedly.

The American pioneer in blood banking, John Elliott, is credited with suggesting in 1936 that plasma be used as a whole-blood substitute. Elliott, along with the Baxter Corporation, developed the first sterile, enclosed glass vessel (TRANSFUSO VAC), containing sodium citrate anticoagulant, for the collection and storage of blood and plasma. The US Army elected to use human plasma for volume replacement in the field, but chose dried plasma over the liquid product because of ease of storage and shipment. The use of plasma as a red cell substitute was eclipsed by advances in collection and storage of whole blood, however.

While plasma never achieved the status of a 'red cell substitute,' it was effective. By the time Amberson reviewed the field in 1937, successful exchange transfusions in dogs with either plasma or serum were being demonstrated routinely. The use of plasma in treating massive bleeding was an accepted procedure. Referring to the studies of the toxic effects of plasma and serum, Amberson stated:

> We feel that plasma colloids, both protein and fat, probably exercise their major effect by maintaining the colloidal osmotic pressure of blood … the normal colloids may be almost completely replaced by other colloids, without injury to the mammalian body, if there be no oxygen lack. Oxygen lack undoubtedly occurred in many of the experiments cited above, and the literature is in a state of confusion because of failure to control this and other factors.

As we shall see in the following chapters, some of this confusion still exists.

World War II ushered in the modern era of blood fractionation. Owen Wangensteen, at Minnesota, showed that plasma could be administered directly to humans (Wangensteen *et al.*, 1940; Kremen *et al.*, 1942). Although cases of 'serum sickness' occurred frequently 5–7 days after the infusion, the procedure could be lifesaving in cases of hemorrhagic shock (Dunphy and Gibson, 1943).

In anticipation of wartime need, the National Research Council asked Edwin J. Cohn, of Harvard University, to investigate the question of whether bovine plasma could be made safe for clinical use. Cohn established an effective multidisciplinary research unit at Harvard, and in 1947 he published the results of their exhaustive studies (Cohn, 1947). Using modern protein chemistry methods, including electrophoresis and ultracentrifugation, Cohn showed that most of the adverse reactions were caused by the globulin fraction and that albumin was safe for parenteral use.

Perfluorocarbons

In 1966, Leland Clark and Frank Gollan demonstrated dramatically that a laboratory mouse

Figure 1.2 Liquid-breathing mouse. The mouse is totally immersed in perfluorocarbon (FC-80, butyltetrahydrofuran) which has been saturated with oxygen by bubbling at room temperature. Such a mouse can survive liquid breathing for many hours (From Clark, 1985).

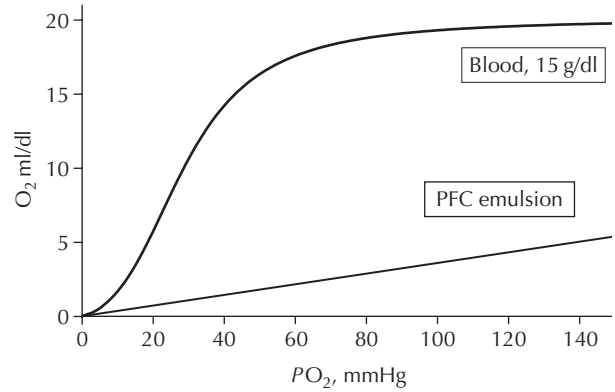

Figure 1.3 Comparison of the oxygen capacity of blood (15 g/dl) and a PFC emulsion (Oxygent™) as a function of PO_2. Note that the tetrameric structure of hemoglobin and its cooperativity lead to nearly complete saturation at the arterial oxygen partial pressure of 100 mmHg.

could survive total immersion in a perfluorocarbon (PFC) solution (Figure 1.2). This material, similar to the commercial Teflon, is almost completely inert, but it is also insoluble in water. Henry Sloviter and Kamimoto prepared a water-soluble emulsion that could be mixed with blood (Sloviter and Kamimoto, 1967), and Robert Geyer and his colleagues were the first to replace completely the blood volume in rats with an emulsion of perfluorotributylamine (Geyer *et al.*, 1968). The animals survived in an atmosphere of 90–100% oxygen, and went on to long-term recovery. However, the oxygen content of the PFCs has a linear dependence on PO_2, and a very high oxygen tension is required to transport physiologic amounts of oxygen (Figure 1.3). This and a propensity to be taken up by the reticuloendothelial cells were considered to be severe limitations to the development of clinically useful PFC blood substitutes (Gould *et al.*, 1986).

Until recently, these problems seemed to present insurmountable hurdles to further development. Now, newer emulsions have been developed that allow higher concentrations of dissolved oxygen, and efforts at developing PFC products were renewed in the 1980s. One product, Fluosol-DA, a 20 per cent (by weight) emulsion, was licensed for use in coronary angioplasty. However, Fluosol-DA did not live up to its early promise because of limited efficacy and a cumbersome packaging system, and it was eventually withdrawn from the market. Newer products that achieve a higher perfluorocarbon content (and hence higher oxygen capacity) have been developed, and are extensively reviewed in this book.

CELL-FREE HEMOGLOBIN

Hemoglobin seems to be the logical choice for a red cell substitute because of its high capacity to carry oxygen (Figure 1.3) and its oncotic properties. Furthermore, hemoglobin is the natural oxygen carrier contained within the red blood cell, so its isolation, purification and use as a substitute for red cells seemed a good idea since it would be free of the limitations of red cells – including the need to cross-match donor with recipient, and restrictions on storage. Von Stark (1898) was probably was the first to treat anemic patients with hemoglobin solution. Although his results were encouraging, he was not able to prepare stable solutions and did not pursue the studies further. Better preparations were reported by Sellards and Minot (1916). They administered very small amounts of hemoglobin in an effort to discover its renal threshold, and reported no untoward reactions in 33 subjects.

Many attempts to administer hemoglobin solutions to humans took place after the reports of

Sellards and Minot, but these are difficult to evaluate because the experience was mixed. Many patients did well, but others demonstrated hypertension, bradycardia, oliguria, and even anaphylaxis. These adverse effects were not correlated with specific biochemical properties of the solutions themselves.

Modified hemoglobin

The first experience with hemoglobin as a red cell substitute made clear that the red cell serves important functions; among them the prevention of rapid elimination of hemoglobin via the kidneys, and rapid breakdown to methemoglobin (the inactive form of the protein). Nevertheless, interest in hemoglobin-based red cell substitutes remained extremely high, particularly in wartime, but the rapid clearance and toxicity in the kidneys had to be overcome. This problem was solved when H. Franklin Bunn, working in the US Army Blood Laboratory at Fort Knox, discovered that crosslinking with bis(N-maleimidomethyl) ether (BME) prolonged its plasma retention (Bunn and Jandl, 1968). Bunn and J. H. Jandl concluded that this was because of a reduced tendency to form dimers in the crosslinked hemoglobin, and therefore the hemoglobin was not filtered by the kidney. Accordingly, they showed that most of the hemoglobin could be found in various tissues rather than in the urine.

Another property of cell-free hemoglobin is its high affinity for oxygen relative to hemoglobin contained within the red cell. The affinity is so high, in fact, that it was feared that little of the bound oxygen would be released in tissue capillary beds. On the assumption that cell-free hemoglobin and red cell hemoglobin should have the same affinity for oxygen, Ruth and Reinhold Benesch employed agents that could react at the 2,3-diphosphoglycerate (2,3-DPG) binding site and reduce the affinity for oxygen (Benesch and Benesch, 1967). This discovery led to other modifications of hemoglobin that could not only reduce its affinity but also stabilize the tetrameric structure so that its vascular retention could be prolonged. The most widely used of these agents was pyridoxal 5′-phosphate (PLP) (Benesch *et al.*, 1972). Viewed in retrospect, it is now astonishing that this assumption was so strong and that it is so deeply ingrained. Tissue PO_2 can fall to just a few mmHg without engaging anaerobic metabolism, and virtually any oxygen would be released, regardless of the affinity of the carrier.

Finally, even exceedingly small amounts of stromal (cell membrane) contaminants in hemoglobin solutions appeared to be toxic. In the 1960s many workers believed that contradictory toxicity reports could be explained by contamination of the solutions with foreign materials. S. Frederick Rabiner studied novel ways to remove stroma from red cell hemolysates (Rabiner *et al.*, 1967) and coined the phrase stroma-free hemoglobin (SFH). His methods included filtration techniques that could be applied to large volumes of hemolysate and made possible physiologic studies in large animals. Rabiner's results gave new hope to the sagging field because they indicated that the toxic effects of hemoglobin might be prevented by rigorous purification.

After the work of Rabiner and colleagues, several 'pure' hemoglobin solutions were produced on a large scale for experimental use. Frank DeVenuto and his colleagues at the Letterman Army Institute of Research described a procedure for 'crystallization' of hemoglobin, and evaluated the product in a series of animal trials (DeVenuto *et al.*, 1979a, 1979b; DeVenuto, 1982; DeVenuto and Zegna, 1981, 1982). The Biotest Serum Institute, Federal Republic of Germany, produced a 6-g/dl hemoglobin solution that had a $P50$ (the PO_2 at which hemoglobin is half-saturated with oxygen) of about 18–20 mmHg and was used in studies of tissue distribution (Bonhard, 1975a). The Warner-Lambert Research Institute in the United States produced a similar solution of SFH, which was used for many basic studies of oxygen transport (Biro *et al.*, 1978) and for a clinical trial in humans.

Payne (1973) described protein polymerization with the tissue fixative glutaraldehyde. Soon, a process for polymerizing hemoglobin with the agent was patented by Laver (Laver *et al.*, 1975), and this material demonstrated a markedly prolonged intravascular retention. Although the reaction is extremely difficult to control, products for infusion were developed (Bonhard, 1975b; Sehgal *et al.*, 1979; DeVenuto and Zegna, 1981). The most successful of these (PLP-polyhemoglobin) is first reacted with PLP and then polymerized with glutaraldehyde; it was the first modified hemoglobin to be used in published human trials (Moss *et al.*, 1989).

Many preparations of modified hemoglobin now have been tested in animals and humans. It appears that most are efficacious in transporting

oxygen, but it has not always been easy to relate toxic side effects to specific structural or functional properties of the molecule. A variety of modified hemoglobin solutions have been studied, including those stabilized with various types of crosslinkers. Other products are derived from hemoglobin conjugated to synthetic materials such as dextran or polyethylene glycol. Sources other than outdated human blood have also been investigated, including cow and recombinant hemoglobins produced in bacteria, yeast, and even transgenic mammals.

Encapsulated hemoglobin

Since hemoglobin is normally packaged inside a membrane, it seems intuitive that encapsulated hemoglobin would be the ultimate solution for the red cell substitute problem. In 1957 Thomas Chang reported the use of microencapsulated hemoglobin as artificial red blood cells (Chang, 1988). Since that time, dramatic results have been reported in the complete exchange transfusion of laboratory animals (Djordjevich *et al.*, 1985; Hunt *et al.*, 1985; Rudolph *et al.*, 1998), but progress toward development of an artificial red cell for human use has been slow because of problems of reticuloendothelial and other macrophage stimulation (Rabinovici *et al.*, 1989). Other problems include: maintaining sterility, endotoxin contamination, cumbersome production requirements, and high cost.

Synthetic heme

Synthetic compounds have been produced which bind or chelate oxygen. These compounds are commercially attractive because their manufacture and licensure may qualify them as drugs, rather than as biologics. Thus, Tsuchida and his colleagues have shown that synthetic heme can be used to transfuse animals (Tsuchida *et al.*, 1988). Synthetic oxygen carriers would solve the problem of a limited supply of hemoglobin for modification; at present, however, the synthetic procedures are very tedious, and the possibility of scale-up seems remote.

CURRENT STATUS

Several of the products mentioned above are now under intense development. A number of perfluorocarbon- and hemoglobin-based products have been approved by the FDA for clinical testing, and several have reached Phase III. A hemoglobin-based product, HemAssist™ (Baxter Healthcare), held great promise, only to be discontinued after a disappointing trial in trauma patients. A perfluorocarbon emulsion, Oxygent™ (Alliance Pharmaceutical), also seemed likely to succeed, only to be discontinued after the unexpected finding of increased risk of stroke in cardiopulmonary bypass patients. Many of the problems described by Amberson have been solved, but others have emerged. This should perhaps not be surprising, since replacement of the red blood cells with massive amounts of protein free in solution is an unprecedented therapeutic adventure. Enthusiasm and despair seem to follow an undulating pattern in this field: progress always seems to reveal new difficulties, and the resulting research always seems to lead to new advances.

Recognizing the magnitude of the blood substitute problem, and in view of the many failures, many scientists have turned their attention to fundamental questions of oxygen transport and examined some of the early assumptions regarding required properties of the solutions. In particular, research has focused on understanding how oxygen is regulated in the microcirculation, and it seems likely that progress in this area may provide the final needed information in order to at last bring an 'artificial' oxygen carrier into clinical use.

SUMMARY

This brief review of the history of blood transfusion serves to remind us of and underscore the importance of timing and context in achieving major milestones of scientific progress. Clearly, without understanding coagulation, infection and blood types, implementation of blood banking and transfusion would not be possible, no matter how much energy, manpower and funds were expended. At the time these discoveries were being made, no one could predict what additional hurdles waited just ahead. Therefore, while presenting a concise history of any scientific discovery imparts a sense of logic and order on the process, in fact it is rather chaotic. In the case of red cell substitute research, the same is true. Each new solution is developed by scientists who believe that all major problems are solved, and that the key discoveries

that led to each product are the last obstacles to be overcome. It is only when unexpected experimental results, whether in animals or humans, are encountered that we realize that a new set of problems needs solving. As the field of red cell substitutes is described in the following chapters, we will try to grasp whether or not additional gaps in our scientific knowledge will prevent products from clinical use in the foreseeable future.

EDITOR'S SUMMARY

No scientific or clinical breakthrough is independent of developments in allied disciplines. The quest for a substitute for blood started when the circulation was discovered by William Harvey in the seventeenth century, but before the goal could be achieved countless details about the function of the normal circulatory system and the function of blood and, particularly, red blood cells remained to be discovered. The history of blood transfusion is long and colorful, marked with attempts to infuse various materials, often with disastrous results. The problem with these early attempts at blood transfusion was that almost nothing was known about the way blood works, only that it circulates. Blood transfusion could not be successful until blood groups were appreciated, anticoagulants discovered and control of infection achieved.

The development of a blood substitute is far more complicated than blood transfusion because it requires understanding of the many mechanisms that come into play to ensure tissue blood flow and perfusion. Some of these are still in question, such as the role of the endothelium-derived relaxing factor, nitric oxide, other endothelium-derived cytokines, neural regulation and oxygen autoregulation in the maintenance of vascular tone. It is not surprising that infusion of an oxygen carrier whose supply of oxygen to tissue is not identical to that of the red blood cell will engage responses that are unpredictable and even detrimental.

The technology to produce oxygen carriers with nearly any set of characteristics is now available. The critical question is whether or not we know enough about the circulatory system to predict which properties are essential and which ones are detrimental.

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Chapter 2

Transfusion Medicine

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This article was written in a personal capacity and does not necessarily represent the opinions or endorsement of the NIH, the DHHS, or the Federal Government

HISTORY OF BLOOD TRANSFUSION

Since the seventeenth century and the first transfusion of blood from sheep to man, the history of blood transfusion has been marked by high expectations, unexpected complications, and impressive advances in lifesaving supportive care. The first successful indirect human transfusion, using a syringe to transfer blood from donor to recipient, is attributed to the English obstetrician James Blundell in1818. Blundell cautioned that transfusion should be reserved for patients *in extremis*. Mortality in his series approached 50 per cent, although the mortality related to transfusion of incompatible blood remains unknown.

The era of modern transfusion medicine dates to the early twentieth century, with the identification of the blood groups A, B, and O by Landsteiner in 1901. Subsequent use of this technique for compatibility testing improved blood safety dramatically, although recognition of the immunologic diversity of red blood cells and sophisticated crossmatching had to await the discovery of the direct antiglobulin (Coombs) test almost 50 years later (Coombs, 1945). The development of anticoagulant (1914) and preservative solutions (1916) led to the establishment of World War I blood depots in British Casualty Clearing Stations, precursors of the modern blood bank, where blood could be stored after collection for several days prior to use (Robertson, 1918). The quality of these cells was not documented, but by all reports these early transfusions saved lives (Hess and Schmidt, 2000). Ironically, no controlled studies of the effectiveness of whole blood or red cell transfusions have ever been conducted, and no trials are likely to be done.

The era of component therapy dates from the development of the plastic blood bag in 1953 and the development of interconnected systems of bags in the 1960s, which not only permitted efficient use of collections from a single donor, but also decreased the risks of bacterial contamination and volume overload from transfusion (Walter, 1984). At about the same time, transfusion-transmitted hepatitis, recognized since World War II, was reported as an inordinately high risk in recipients of blood from commercial sources (Allen, 1959). As a result, developed countries have almost universally instituted all-volunteer blood donation systems. However, the challenges of avoiding pathogen transmission through blood have moved to the forefront of blood safety, virtually dwarfing concerns about other risks and about a dwindling supply of blood and components.

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ORGANIZATION OF BLOOD SERVICES

Most developed countries have a single national blood provider, some organized within the governmental framework (like the National Blood Service in the United Kingdom) and others through a single non-governmental provider (such as the Red Cross Blood Program in Australia). Still other countries have moved from a single non-governmental provider to one that is overseen by government ministers, such as the Canadian Blood Service, which was reorganized in 1998 to effect tighter governmental control and yet still allow the Canadian Red Cross to recruit donations of more than 800 000 units a year. The US, Germany, Italy, and a number of countries in South America rely on more pluralistic 'competitive' systems to collect and distribute blood components.

In the United States, the American National Red Cross (ARC) initiated the first nationwide civilian blood program after World War II. Thirty-five Red Cross centers now supply nearly 50 per cent of the blood and blood components in the country. Most of the remaining blood for transfusion is collected by independent not-for-profit community blood centers. In 1962, seven community-based blood centers established the Council of Community Blood Centers, currently known as America's Blood Centers, a federation of independent blood collectors that now stretches from coast-to-coast and supplies more than 40 per cent of US collections. Approximately 8 per cent, or about 1.2 million units, is still collected by hospitals, a figure that has not changed substantially in 20 years (Surgenor *et al.*, 1990; Wallace *et al.*, 1995; AABB, 2003). The rising costs of blood have encouraged several hospital systems to consider opening their own donor collection networks, but increasing federal oversight of this highly regulated industry and the costs of compliance are likely to dampen the enthusiasm of the most ardent small collector.

The American Association of Blood Banks (AABB), established in 1947, is an international, not-for-profit professional association involved in standard setting and the accreditation of facilities that collect and transfuse blood. AABB membership includes approximately 1800 institutions and 8000 individuals, including physicians, medical technologists, nurses, blood donor recruiters and administrators in all 50 states and in 80 foreign countries. AABB collects no blood but has established the National Blood Exchange, a nationwide clearinghouse for AABB institutional members that matches blood surplus with needs. ARC and ABC have internal clearinghouse capability as well. AABB has also joined ARC to establish a single national registry of rare donors to help support patients with difficult transfusion problems.

The US supports a dual system of blood collection. While blood and its components are collected from volunteers in accordance with National Blood Policy (Department of Health and Human Services, 1974), most plasma for fractionation is collected from paid donors by commercial firms. A trade association known as the Plasma Protein Therapeutics Association (PPTA) represents these collectors and the commercial fractionators. Although several countries have undertaken volunteer domestic plasma collection programs, plasma collected in the US remains the cornerstone of commercial protein concentrates around the world.

Most countries oversee quality and compliance of blood collection through a governmental regulatory body. In Australia, the Therapeutic Goods Administration regulates the not-for-profit Red Cross. In Germany, the Paul Ehrlich Institute regulates a pluralistic system of blood providers. In the United States, collection and transfusion practices are governed both by federal statute and regulation as well as by voluntary accreditation. AABB accreditation employs a peer-review process that requires that members adhere to *Standards for Blood Banks and Transfusion Services*, a set of voluntary standards now in its twenty-third printing (Fridey, 2003). The Joint Commission on Accreditation of Healthcare Organizations (JCAHO) and the College of American Pathologists also enforce voluntary standards for transfusion and laboratory practices (College of American Pathologists, 2004; Joint Commission on Accreditation of Healthcare Organizations, 2004). The Food and Drug Administration (FDA), a governmental agency that derives its authority from the Food, Drug, and Cosmetics Act and from the Public Health Service Act, monitors blood collectors scrupulously. The FDA has the responsibility of assuring blood safety, purity, and potency – a daunting task, since each of the 19 million or so components collected each year represents a single 'lot' by regulatory definition. The FDA licenses establishments and products, and inspects facilities for adherence to Good Manufacturing Practises (GMPs). Detailed regulations are published under Title 21 of the *Code of Federal*

Regulations (FDA, 2004), and are updated regularly. The FDA has the authority to close facilities, revoke licensure, and invoke criminal proceedings by the Department of Justice.

AVAILABILITY OF BLOOD

Blood availability has become a safety issue. Mounting concern for blood safety has excluded an increasing number of potential volunteer donors just as the need for blood grows in aging populations around the world.

The blood donor

Qualification of blood donors has become a lengthy and detailed process – a virtual 'donor inquisition'. Blood collection depends on a system of safeguards that include sensitive screening tests; detailed donor education programs prior to recruitment; pre-donation informational literature; stringent donor screening, selection, and deferral procedures; post-donation product quarantine; and donor tracing and notification when instances of disease transmission are detected. Each element plays a role in preventing 'tainted' units from entering the blood inventory. The donor interview is conducted in a setting sufficiently private to permit discussion of confidential information. With current practices in the US, approximately 2 per cent of volunteer donors still conceal risks that would have led to deferral at the time of donation (Glynn *et al.*, 1998). Blood collectors then perform a limited physical examination designed to protect donor and recipient. Screeners assess the donor's general appearance, pulse and blood pressure, body weight and temperature, and examine venipuncture sites for evidence of illicit drug use.

Frequency of donors in the population

Although in many western countries some 60 per cent of the population are healthy adults aged 18–65 years, and thus qualified to be blood donors, Switzerland enjoys the highest annual frequency of donation in the world at only 10 per cent of the eligible population (Linden *et al.*, 1988; Hassig, 1991). The frequency in most underdeveloped countries is less than 1 per cent (Leikola, 1992).

The number of units collected per thousand US inhabitants of usual donor age (18–65 years) was 88.0 in 2001, up from 80.8 in 1999. While this number compares favorably with the rate of 72.2 per thousand in 1997, it pales in comparison with the 100 units per 1000 population collected in Switzerland. As treacherous as it may be to interpret these figures, the numbers suggest that US collecting facilities are progressively improving efficiency. Data from the American National Red Cross indicate that the average volunteer donates about 1.7 times a year. Losses from outdated red cells accounted for 5.3 per cent of the supply, but given the fact that red cells can be transfused only to compatible recipients, the number of usable units outdated appears to be extremely small. More than 99 per cent of group O units and 97 per cent of group A units were transfused (AABB, 2002).

Blood utilization and shortages

Despite the constant rise in collections, blood collectors report frequent shortages, and emergency appeals for blood are disturbingly common. There is a declining margin between US blood collections and transfusions (Schreiber *et al.*, 2003). Globally, approximately 75 million units of blood are collected each year, of which about 45 million units (or about 60 per cent) are collected in developed countries (WHO, 2001). Some 15 million units of red cells and 12 million units of platelets were collected in the US in 2001 – an increase of more than 1 million units from the number reported in 1999, the last previous year for which national blood data were assembled. Autologous units accounted for only 2.6 per cent of units transfused, and fewer than 150 000 units were 'directed' to specific patients (AABB, 2002). With the current shelf life of up to 42 days, the blood supply more closely resembles a pipeline than a bank or reservoir. A few days of under-collection can have a devastating effect on supply. While most national supermarket chains have developed efficient barcode-based information systems to monitor perishable inventory on a daily basis, few national blood services have as accurate an accounting of blood component location and availability by group and type. Furthermore, there is little general agreement about what constitutes a shortage. Measures of postponed surgery and transfusion, as well as increased rates of RhoD positive transfusions to RhoD negative recipients provide some indication of shortage at the treatment level. In a national survey in the United States in

2001, 138 of 1086 hospitals (12.6 per cent) reportedly delayed elective surgery for 1 or more days, and 18.9 per cent experienced at least 1 day in which transfusion was postponed because blood needs could not be met; a separate government-sponsored study revealed seasonal fluctuations of blood appeals and cancellations of surgery for lack of platelet transfusion support (AABB, 2002; Nightingale *et al.*, 2003).

Individuals 65 years or older constitute 13 per cent of the US population, but receive 25 per cent of blood units transfused (Heinrich, 1999). The US decennial census 2000 projects that by the year 2030, the population of Americans over age 65 will increase from 12 per cent to 20 per cent; this figure will be even higher in most countries in Western Europe (Kinsella and Velkoff, 2002). Given these projections, developed countries may expect blood shortages to become a way of life unless substantial resources are invested in donor recruitment and retention. In developing countries, this is already the case.

The shrinking donor pool: the safety vs. availability balance

Measures introduced to increase blood safety may have the unintended consequence of decreasing blood availability. Results from demographic studies indicate that certain donor groups or donor sites present an unacceptable risk of disease transmission. For example, blood collectors no longer schedule mobile drives at prisons or institutions for the mentally disadvantaged because of the recognized high prevalence of transfusion-transmissible viruses in their residents. Few would argue the risk–benefit analysis of these exclusions. More questionable are the temporary exclusions of soldiers exposed to multiple tick bites at Fort Chaffee, Arkansas. Donors who have received human growth hormone injections have been indefinitely deferred because of the possible risk of transmitting Creutzfeldt-Jakob disease (CJD). However relatives of patients with 'sporadic' CJD are still deferred in the US (except for preparation of plasma fractions) despite evidence of their safety (Center for Biologics Evaluation and Research, 2004). There have now been five case control studies of more than 600 CJD cases, two lookback studies of recipients of CJD products, two autopsy studies of patients with hemophilia, and mortality surveillance of 4468 CJD deaths over 16 years without any link to transmission by transfusion (Center for Biologics Evaluation and Research, 2004). While the impact of this deferral on the US blood supply has been negligible, the recent indefinite deferral of donors who resided in the United Kingdom for a total of 3 months or longer between 1980 and 1996 and the complicated deferral policy for residents and visitors to the European continent, designed to reduce the theoretic risk of transmission of the human variant of 'mad cow disease' (vCJD), has had a substantial impact, particularly on apheresis donors. Estimates of US FDA mandated geographic deferrals based on European residency predict an overall loss of 5–10 per cent of US blood donors, and the impact is expected to vary by region (Center for Biologics Evaluation and Research, 2004). Additional donor exclusions appear to be on the horizon.

A study of 116 165 persons who presented to a regional blood center during the year 2000 indicated that 13.6 per cent were deferred at presentation. Short-term deferral accounted for 68.5 per cent, most commonly for low hematocrit. Long-term deferral, for example for travel to a malarial area, accounted for 21 per cent, and multiple-year or permanent deferral, such as that for vCJD, accounted for 10.5 per cent (Custer *et al.*, 2004). Collection of overweight and underweight units led to a loss of 3.8 per cent of 100 141 collections. More troublesome, though not as large, are donor deferrals resulting from false-positive infectious disease screening tests. Disease-marker-reactive donations represented 0.9 per cent of donation deferrals in the study above (Custer *et al.*, 2004). This problem has been recognized since the introduction of serologic tests for syphilis. However, over the past 15 years the introduction of new screening tests and testing technologies has resulted in numerous deferrals for 'questionable' test results, and either complex re-entry algorithms or no approved method to requalify such donors. Surrogate tests used for screening have proved particularly troublesome (Linden *et al.*, 1988). However, even specific tests result in inappropriate deferrals. Several million donors now fall into this category. Selected donor deferral criteria are listed in Table 2.1.

RED BLOOD CELL COMPONENTS

Whole blood and red blood cells

Whole blood (450–500 ml) is obtained from donors who meet standard donor criteria established by

Table 2.1 Selected donor deferral criteria

Category	Deferral details	Length of deferral
General health	Diseases of heart, liver, lungs; blood disorders	Indefinite
Age	Less than age of majority	Applicable state law
Examination	Hemoglobin < 12.5; BP systolic > 180, diastolic > 100; pulse <50> 100 or irregular; temp. > 37.5°C; any lesions at venipuncture site	Until within range
Medications	Finasteride, isotretinoin	1 month after last dose
	Dutasteride	3 months after last dose
	Acetretin	3 years after last dose
	Etretinate	Indefinite
	Antibiotics and other medications	Physician's discretion
Pregnancy		6 weeks post partum
CJD	Family history, dura mater transplant, human pituitary growth hormone	Indefinite
vCJD	Residence in UK > 3 months between 1980 and 1996; residence in Europe > 5 years since 1980; > 6 months on European military base; received UK bovine insulin; transfused in UK	Indefinite
Transfusion/transplant	Receipt of blood or other human tissue	1 year
Immunizations	Live attenuated viral/bacterial vaccine	2 weeks
	Smallpox vaccine	4 weeks
	Live attenuated rubella/zoster	3 weeks
	Other vaccines/HBIG	1 year
Cancer	Other than basal cell carcinoma	5 years to indefinite
Infectious disease	Residents of malaria endemic areas	3 years
	Travelers to malaria endemic areas	1 year
	History of hepatitis after age 11	Indefinite
	Donated single unit associated with hepatitis, HIV, HTLV	Indefinite
	Tested positive on screening test	Indefinite
	Babesiosis or Chagas hx	Indefinite
	Stigmata or hx of parenteral drugs	Indefinite
	Male sex with males since 1977	Indefinite
	Exchanged sex for money since 1977	Indefinite
	Residence in African countries associated with HIV2	Indefinite
	Persons transfused with blood from such countries	Indefinite
	Sexual contact with persons from such countries	Indefinite
	Treated for gonorrhea or syphilis	1 year
	In jail for > 72 hours	1 year
	Victim of rape or bloody needle stick	1 year

the Food and Drug Administration's *Code of Federal Regulations* and the American Association of Blood Banks *Standards for Blood Banks and Transfusion Services* (Fridey, 2003; FDA, 2004). Whole blood is separated by centrifugation into components such as red blood cells, platelets and plasma, and stored under differing conditions designed to best maintain viability and efficacy of the particular component. Red cells are also collected by automated blood cell separators, either as 'double-unit' red cells or as a single unit along with a plasma or platelet component (Bonomo *et al.*, 2004). In the US, whole blood is infrequently used and thus rarely available in

most institutions. The few settings in which whole blood transfusion is indicated include hemorrhage with acute hypovolemia, massive transfusion, and exchange transfusion if done by manual phlebotomy rather than by apheresis. Whole blood, as well as other components, is infused routinely through a 170–260-micron infusion filter to remove clots.

Red blood cells (RBC) are separated from whole blood shortly after collection, and are stored in a volume of 180–200 ml at refrigerated temperatures for 21, 35 or 42 days, depending on the country and the anticoagulant-preservative used. RBC transfusion is indicated for the treatment of symptomatic anemia that cannot be managed by other means, for example by replenishing such deficiencies as B12, iron, or folic acid. The concept of the 'transfusion trigger' is a controversial one, since RBC transfusion should be based on careful assessment of a patient's clinical symptoms and coexisting or medical conditions, as well as laboratory determination of hematocrit and hemoglobin (Consensus Conference, 1988; American Society of Anesthesiologists, 1996). Normal subjects at rest can tolerate severe anemia without cardiovascular compromise; however, anemia appears to be a particular risk factor for patients with cardiovascular disease receiving intensive care and may require more aggressive transfusion management (Weiskopf *et al.*, 1998; Hebert *et al.*, 1999). The number of patients who suffer cardiovascular events from under-transfusion is not known, but studies of such diverse patient groups as children with malaria, patients who decline transfusion for religious convictions, and elderly patients with acute myocardial infarction suggest that the number may be substantial (Nelson *et al.*, 1993; Carson *et al.*, 1996; English *et al.*, 2002; Wu *et al.*, 2003). In general, transfusion of 1 unit of red blood cells will increase the hematocrit by 3 per cent or the hemoglobin by 1 gm/dl in an average-size adult. In the average pediatric patient, transfusion of 8–10 ml/kg of red blood cells is expected to increase hemoglobin by 3 gm/dl.

RED CELL COMPATIBILITY

Serologic compatibility testing has been used for almost a century to prevent antibodies in the patient's plasma from destroying transfused RBC (Ottenberg and Kaliski, 1913). While the earliest tests involved mixing of donor and recipient whole blood, incubation, and observation for agglutination or hemolysis, this technique detected only ABO blood group incompatibility. A variety of additional methods, using albumin as the cell suspension medium and an antiglobulin (Coombs) reagent to detect non-agglutinating antibodies such as those of the Rh, Kell, Kidd, and Duffy systems, is now used in pre-transfusion testing (Coombs *et al.*, 1945; Fridey, 2003). Compatibility testing now involves testing of donor blood for ABO/Rh antigens, testing the donor's plasma for antibodies, testing the recipient cells for ABO/Rh and the recipient plasma for antibodies, 'cross-matching' donor cells with patient plasma, and reviewing previous testing records. The process takes about 1 hour. When emergency transfusion is necessary, several abbreviated strategies have been recommended. Since most fatal reactions are caused by incompatibilities in the ABO system, ABO typing alone (5 minutes) or with an 'immediate spin crossmatch' (total 20 minutes) shortens the procedure. The complete compatibility protocol is performed subsequent to transfusion. For the most urgent cases, group O RhoD-negative or even RhoD-positive blood is used and compatibility testing is completed after the fact. Use of O-negative ('universal donor') blood has been advocated for ambulance and field use for trauma, but providing sufficient suitable refrigerated units has posed a logistical challenge.

When the plasma screen for alloantibodies is positive, antibody specificity must be determined and the corresponding antigen avoided in red cell transfusions. The process of identification can be lengthy or require referral to a specialized red cell reference laboratory. If a clinically important alloantibody is directed against a very high frequency antigen (present in greater than 90 per cent of individuals) or when multiple alloantibodies are present, procurement of compatible blood may be difficult or impossible. Rare donor registries are available to assist in selected instances. In some cases, the presence of red cell autoantibodies makes all units (including those of the patient) appear incompatible and masks the presence of alloantibodies. A negative antibody screen does not necessarily indicate absence of alloantibodies, since the titer of antibody may fall below the level of detection or the antibody might be directed against a low incidence antigen (present in less than 10 per cent of individuals) not present on reagent testing cells, yet present on some patient cells. Weakly

reactive specimens do not predict that hemolysis will be mild.

UNIVERSAL RED CELLS

Accidental transfusion of ABO-incompatible RBC is a leading cause of fatal transfusion reactions. Studies have estimated a 2.2 per cent error rate in drawing phlebotomy specimens from hospital patients (Renner *et al.*, 1993). Between 1990 and 1998, the New York State Department of health reported that 1 of 19 000 RBC units of incorrect ABO or Rh group was issued or administered to other than the intended patient. Half of these events occurred outside the blood bank (administration to the wrong recipient, 38 per cent; phlebotomy errors, 13 per cent). Isolated blood bank errors, including testing of the wrong specimen, transcription errors, and issuance of the wrong unit, were responsible for 29 per cent of events. Many events (15 per cent) involved multiple errors (Linden *et al.*, 2000). The risk of fatality related to acute hemolytic transfusion reaction, most often caused by ABO incompatibility and erroneous administration, is estimated between 1 : 600 000 and 1 : 1 800 000 (Linden *et al.*, 1988; Renner *et al.*, 1993).

Some hospitals have addressed the problem of compatibility errors by restricting blood in the emergency room and surgical suites to type O. Unfortunately, type O represents only 45 per cent of collections in the US and in most developed countries. One strategy to reduce the risk of incompatible transfusions and to create a universal blood supply involves converting blood group A and B antigens to H, using specific exo-glycosidases capable of removing the immunodominant sugar residues (Olsson *et al.*, 2004). Conversion of group B red cells to O, initially carried out with alpha-galactosidase extracted from coffee beans, resulted in a successful phase II trial utilizing recombinant enzyme (Kruskall *et al.*, 2000). Enzymatic conversion of group A red cells has lagged behind due to lack of appropriate glycosidases and the more complex nature of A antigens (Olsson *et al.*, 2004). A universal red cell might eliminate many transfusion errors and simplify inventory control and complex shipping practices.

RED CELL MODIFICATION

Blood components are often modified for special purposes, and such modifications may result in changes in composition and storage characteristics of the components as well as significant increases in costs (Table 2.2). Some of the commonly used procedures include leukocyte reduction to remove excess white blood cells that may cause alloimmunization and febrile reactions, gamma irradiation to inactivate contaminant lymphocytes responsible for transfusion-associated graft-versus-host disease, washing to remove plasma and plasma proteins that may cause allergic reactions, and cryopreservation for long-term storage of rare blood types. Any modification or manipulation that results in interruption of the closed container of a blood component (such as washing and some methods of deglycerolization or thawing) will affect the component's expiration date. Once the closed seal is broken, refrigerated storage is limited to 24 hours.

Other considerations

Whole blood and red cells, unlike many drugs and biologics, are particularly sensitive to medications and solutions that may be infused concurrently through the same intravenous line. Red cells should be infused with isotonic solutions, and not with hypertonic or hypotonic solutions. Simultaneous infusion with solutions containing glucose or calcium, such as D5W, and lactated Ringer's solution, may result in agglutination or hemolysis of the cells. Simultaneous infusion of medications should be avoided unless the medication has been demonstrated to be compatible with red cells. Fine-gauge needle and pump infusion may also result in hemolysis. Transfusion of red cells should not exceed 4 hours. The quality of the cells and the risk of bacterial growth increase with increasing time at room temperature. If transfusion is anticipated to take longer than the standard allotted 4 hours, the unit should be divided into smaller aliquots.

Red cells must be stored in monitored refrigerators, and Good Manufacturing Practices require recording of temperature and validation of storage conditions. This requirement makes use of satellite refrigerators in emergency rooms and operating suites logistically difficult. If refrigerated blood is issued but infusion is not started within 30 minutes, the component should be returned to the blood bank or discarded. If it is necessary to warm chilled units, only approved warming devices should be used. Unmonitored water baths and microwave ovens have caused

Table 2.2 Indications for modifications of red cell components

Indication	Description	Purpose	Indications	Comments
Leukoreduction	Filtration of component after collection, at bedside, or removal of WBC during automated collection for a 3-log reduction (99.9 per cent) of WBC; final WBC content $\leqslant 5 \times 10^6$	Reduction of febrile nonhemolytic transfusion reactions (FNHTR); reduction of CMV transmission (CMV-safe); reduction of HLA alloimmunization	Patients who have experienced an episode of FNHTR; alternative to CMV-negative components (from donor tested negative for CMV); neonates; transplant patients	Equivalent to CMV seronegative components; *not effective and not indicated for prevention of transfusion-associated graft-versus- host disease*
Irradiation (red blood cells, platelets, granulocytes)	Gamma irradiation (cesium or cobalt) of component with 2500 cGy (centigrays) to inactivate viable lymphocyte proliferation within the component	Prevention of transfusion-associated graft-versus-host disease	Recipients of allogeneic hematopoietic or solid organ transplants; recipients of transfusion from blood relatives; patients on immuno-suppressive regimens; patients with congenital immunodeficiencies and certain malignancies; premature infants (especially those undergoing extracorporeal membrane oxygenation)	*Not* indicated for prevention of FNHTR and unnecessary for aplastic anemia patients (despite ATG therapy) or HIV patients in the absence of other indications for irradiation (above); RBC shelf-life is decreased to 28 days (if greater than the original expiration date)
Washing (plasma removal)	Component washed with sterile normal saline to remove $>$98 per cent of plasma proteins, electrolytes, and antibodies; WBC content 5×10^8	Prevention of allergic reactions; decrease risk of hyperkalemia	Patients who experience recurrent severe allergic reactions (not responsive to premedication with antihistamines); IgA deficient patients when IgA- deficient component is not available; recipients at risk from hyperkalemia; newborns; intrauterine transfusions; may be effective when ABO identical blood is not available for patients with paroxysmal nocturnal hemoglobinuria (PNH)	Washing results in a 15–20 per cent loss of red cells; *not* equivalent to 'leukoreduced'; washed red cells must be transfused within 24 hours

(*Continued*)

Table 2.2 Indications for modifications of red cell components (*Continued*)

Volume reduction	Removal of plasma from cellular components; most RBC concentrates contain very little plasma	Reduction of circulatory overload; removal of antibodies	*Not* equivalent to washing for prevention of allergic reactions in patients who are plasma volume expanded; normovolemic chronic anemia; thalassemia major; sickle cell disease; congestive heart failure; pediatric, elderly, and other patients susceptible to volume overload	
Freezing-deglycerolization	Addition of glycerol and freezing generally within 6 days of collection depending on the additive solution used at the time of collection and glycerolization-freezing method used	Long-term storage of autologous or allogeneic rare blood phenotypes	Patients with rare blood phenotypes or multiple alloantibodies	May *not* be feasible for red blood cells with certain abnormalities (such as Hgb S, hereditary spherocytosis, PNH); *not* equivalent to 'leukoreduced' *(may remove > 95 per cent of WBC)*; depending on the method of glycerolization-freezing used (open or closed system), the post deglycerolization shelf-life may be 24 hours or 2 weeks (respectively); thaw–wash process is lengthy – this component is not suited to emergent supply of multiple units

hemolysis of red cells, which can prove lethal to the recipient.

ADVERSE EVENTS ASSOCIATED WITH RED CELL TRANSFUSION

Disease transmission

Detailed donor screening and specific testing of blood for transfusion have improved blood safety dramatically. The current multi-layered approach relies on donor education, donor screening, testing, registries of previously deferred donors, post-donation tracking and, in several countries, universal transfusion surveillance (hemovigilance) (Busch *et al.*, 1999). Even though the blood supply has become remarkably safe by historical standards, the tragic experience with HIV and HCV transmission fuels ongoing public concern with blood safety and intense scrutiny of the organizations responsible for the provision of blood and its components (Klein, 2000).

Currently in the US, blood safety relies upon a system of voluntary donation, a detailed donor history, and a variety of serologic and nucleic acid-based (NAT) tests including assays for HIV I/2, HTLV I/II, hepatitis C, hepatitis B, West Nile virus, and *Treponema pallidum* (the agent that causes syphilis). This approach has proved effective. With improved screening, most new transmissions now occur as a result of the 'window period' interval between the time the donor is infected and the moment at which tests are capable of detecting the agent. Increased sensitivity of screening tests has gradually closed this window (Table 2.3).

The residual risk from repeat donors approximates 1 : 144 000 for HBV, 1 : 1 935 000 for HCV and 1 : 2 135 000 for HIV, although rates for these agents are some two-fold greater among first-time donors (Dodd *et al.*, 2000). The rates are even lower in much of Europe, reflecting the lower prevalence in the general population (Pillonel and Laperche, 2004). No transfusion-transmitted case of syphilis has been reported in the US in more than 30 years (Schmidt, 2001). Nevertheless, infectious risks remain. The rate of transfusion-transmitted bacteremia is conservatively estimated at 0.21 per million units of RBC transfused (Kuehnert *et al.*, 2001). Culture-based testing remains cumbersome even for platelets, and has not been applied to red cell components (Brecher and Hay, 2004). A number of tick-borne diseases, such as babesiosis and human granulocytic ehrlichiosis, have been transmitted by blood, and others such as Lyme disease have that potential (Cable and Leiby, 2003). Some common infectious agents, such as cytomegalovirus and parvovirus B19, are readily transmitted by blood, and though often innocuous may be devastating for particularly vulnerable patients such as pregnant women, premature infants and immunosuppressed patients. Most blood components are not tested for these agents. Perhaps the greatest worry in the developed world is the silent emergence of some new agent, like HIV in the 1980s, for which recognition (and therefore testing) may be delayed for years while asymptomatic carriers donating blood spread infection through the blood supply. The most recent example involves emergence of the West Nile virus, an agent that infected thousands of blood donors, resulted in transfusion-transmitted disease, and presented a sudden, unexpected and significant threat to the US blood supply (Pealer *et al.*, 2003). Methods to inactivate infectious agents have been successfully applied to plasma fractions, but no technique has proved both safe and effective for red cells.

Table 2.3 Estimated risk of transfusion transmission of viral agents

Virus	Risk per unit
HIV 1 and 2	1 : 2 000 000–3 000 000
Hepatitis B	1 : 1 00 000–200 000
Hepatitis C	1 : 1 000 000–2 000 000
HTLV I and II	1 : 641 000 (cellular components)
Parvovirus B19	1 : 20 000 (most recipients immune)
West Nile	Range 1 : 10 000 to 1 : 150, depending on region of epidemic
Bacterial contamination	1 : 5 000 000

The situation is worse in the developing world. More than two-thirds of the world's nations do not have adequate policies to insure a safe blood supply. An estimated 13 million blood donations globally are not tested for HIV, HBV and HCV, primarily in developing countries where the number of infected persons in the donor population is high (Sarkodie *et al.*, 2001; Dhingra *et al.*, 2004). Furthermore, areas in the world in which HIV, HCV, and HBV are endemic would have little blood available even if universal blood testing were logistically and economically

feasible; 20 per cent or more of the population might test positive. In Asia, Africa and parts of Latin America, diseases such as malaria, trypanosomiasis and leishmaniasis, almost forgotten in the US and Europe, remain important transfusion-transmitted diseases with substantial morbidity and mortality.

Other adverse events

Although public concern about blood safety centers on the risk of transfusion-transmitted infection, red cell transfusion carries a number of other potential risks, both immunologic and non-immunologic. The risk of acute hemolysis, primarily from erroneous transfusion of ABO incompatible units, has been mentioned previously. Delayed hemolytic reactions caused by alloantibodies directed at other blood group antigens occur in approximately 1 : 4000 units of red cells transfused (Moore *et al.*, 1980). While most of these reactions are clinically mild, intravascular hemolysis with renal failure, disseminated intravascular coagulation and death have been reported. Non-immune hemolysis can also result from units of red cells that are overheated, frozen, or mixed with hypotonic solutions.

Chills and fever resulting from red cell transfusion are common. Of nearly 100 000 units of whole blood and red cells transfused from one blood center in 1980, approximately 1 per cent reportedly resulted in a febrile reaction, and 15 per cent of recipients who were subsequently transfused experienced a second episode of fever (Menitove *et al.*, 1982). This oft-cited statistic is undoubtedly too conservative because of an underreporting bias in the study design. A prospective study of 531 HIV-infected and AIDS patients who received 3864 red cell units during 1745 transfusion episodes documented the frequency of fever as 16.8 per cent (Lane *et al.*, 2002). Fever associated with transfusion was *recorded* about four times as often as the hospital attending staff *reported* it using a voluntary transfusion reaction form. Fever exceeding 2°C occurred in 3.1 per cent of recipients.

The role of leukocytes in causing transfusion reactions is well documented. Severe reactions are characterized by flushing within 5 minutes of the start of transfusion, and a sensation of warmth followed by a temperature spike and rigors about 60 minutes after the start of the transfusion (Brittingham and Chaplin, 1957). While these reactions are often classified as insignificant by attending staff, they are of great concern to the patient and result in substantial delays in transfusion and costs for evaluation of the fever.

A related, but far more severe, pulmonary reaction has been associated with leukocyte antibodies in donor blood. The typical reaction is characterized by chills, fever, a non-productive cough, dyspnoea, cyanosis, and hypotension or hypertension occurring within an hour or two of transfusion (Popovsky and Moore, 1985). Characteristic chest radiographic findings include bilateral pulmonary infiltrates and numerous, predominantly perihilar opacities; and infiltration of the lower lung fields without cardiac enlargement or engorgement of the vessels is described. Unlike pulmonary edema associated with circulatory overload, central venous pressure and pulmonary wedge pressure are not elevated with transfusion-related acute lung injury (TRALI). Some 15 per cent of cases present with mild to moderate hypotension, typically unresponsive to fluid challenge, while another 15 per cent present with hypertension (Popovsky and Haley, 2001). In the United States, TRALI has been estimated to occur after about 1 : 5000 blood component transfusions (Moore *et al.*, 1980). While TRALI is more frequent with plasma transfusion than with red cell components, it has been described with all blood components, and as little as 2 ml plasma seems to be sufficient to cause respiratory distress.

Transfusion-associated graft-versus-host-disease (TA-GvHD) occurs when immunocompetent allogeneic lymphocytes in a blood component engraft in the recipient, proliferate, and mount an attack against the host tissues. TA-GvHD disease occurs between 4 and 30 days after transfusion. When the full-blown syndrome is observed and multiple organ systems are involved, mortality is high, approaching 90 per cent (von Fliedner *et al.*, 1982). However, less severe disease, especially if transient, may not be recognized, much less reported. TA-GvD has been observed after transfusion of whole blood, packed red cells, platelet concentrates, granulocyte concentrates and fresh plasma (Anderson and Weinstein, 1990). In order to avoid the risk of GvHD from blood transfusion, the transfused components must be irradiated to inactivate donor T lymphocytes. In Japan, a country in which there is less genetic diversity than in the US, almost all blood components are gamma irradiated.

Post-transfusion purpura (PTP) is a well-recognized if uncommon syndrome, in which

patients with platelet-specific alloantibodies develop profound thrombocytopenia between 2 days and 2 weeks after a transfusion (Pegels *et al.*, 1981). Purpura, epistaxis, gastrointestinal bleeding and hematuria characterize the clinical syndrome. If untreated, the course may be mild and self-limited, or hemorrhage can be dramatic; mortality is reported as 5–10 per cent.

The frequency of severe anaphylactic reactions following transfusion is about one case per 20 000 transfusions. Class-specific anti-IgA has been detected in 76.3 per cent of 80 IgA-deficient patients with a history of anaphylactic transfusion reaction. Of these patients, 48 received 525 components drawn from IgA-deficient donors without clinical reaction (Sandler *et al.*, 1996). In contrast to anaphylaxis, urticaria during transfusion is relatively common. The incidence has been approximated at 3 per cent (Stephen *et al.*, 1955). In a retrospective analysis of 1613 transfusion reactions during a 9-year period at a single institution, allergic reactions accounted for 17 per cent; however, of these only 7 per cent were severe, accounting for 1.7 per cent of all transfusion reactions. In a prospective study of platelet transfusion, extensive urticaria occurred during 0.4 per cent of transfusions and in 4.4 per cent of patients (Enright *et al.*, 2003). Allergic reactions occurred with a frequency of 1 : 4124 blood components and 1 : 2338 transfusion episodes (Domen and Hoeltge, 2003).

Hypotensive reactions, occasionally severe enough to require circulatory support, have been encountered when bradykinin components and angiotensin are generated as plasma is exposed to charged synthetic surfaces. After a series of reports of hypotensive reactions in 1993, an American Association of Blood Banks survey reported 17 hypotensive reactions, the vast majority of which began within an hour of transfusion and were associated with blood administration through a filter used to remove leukocytes (Dhume *et al.*, 1996). Filtered whole blood and RBC transfusions have been implicated. Subsequent reports implicated filters from several different manufacturers. All of the different blood components passed through these filters at the bedside may result in hypotension; the mechanism appears to be generation of labile vasoactive proteins such as bradykinin. In a study of patients undergoing orthotopic heart transplantation, 24 episodes of hypotension in were observed in 30 patients (47 per cent of patients) who received components filtered at the bedside. Eleven of these patients (79 per cent) were receiving ACE inhibitor medication (Lavee and Paz, 2001).

IMMUNOMODULATORY EFFECTS

Numerous alterations in circulating blood cells have been reported in patients transfused with allogeneic blood. These changes include decreased numbers of circulating lymphocytes, modifications in the T-cell helper/suppressor ratio, changes in B-cell function, down-regulation of antigen-presenting cells, and activation of immune cells as measured by a number of cell surface markers (Klein, 1999). Some of these changes persist for months or even longer after transfusion. The lingering question has been whether these observations represent no more than laboratory curiosities, or whether they reflect some clinically relevant alteration in the recipient's immune status.

The seminal publication by Opelz *et al.* in 1973 provided clinical evidence that, contrary to the conventional wisdom, blood transfusion prior to renal transplantation improved the survival of cadaver-derived renal allografts (Opelz *et al.*, 1973). Furthermore, the effect appeared to be dose-dependent. While this was one of the first clinical observations suggesting that blood transfusion might have an immunomodulatory or tolerizing effect, the role of perioperative blood transfusion in the recurrence of surgically excised tumors and in the survival rates of cancer patients has now been disputed for several decades (Blajchman and Bordin, 1994). Similar controversy surrounds the relationship between perioperative transfusion and the risk of postoperative infection. Several trials suggest that fewer postoperative infections and several-fold reductions in rates of morbidity and mortality occur if leukocytes are removed by filtration from the transfused red cells (Jensen, 1998). Based on the sum of evidence, immunomodulation seems likely to be added to the list of unintended effects of allogeneic blood transfusion. The magnitude and importance of these effects, the causative agents, and the patients or patient groups that are at particular risk have yet to be defined (Klein, 1999).

Massive transfusion of red cells can result in a variety of metabolic abnormalities, including hypocalcemia from the citrate load, hyperkalemia,

and acidosis. Rapid infusion of cold blood has also been associated with arrhythmias.

THE COST OF RED CELLS

The cost of a unit of red blood cells has been notoriously difficult to calculate. Like most commodities, the cost of a unit of blood varies from region to region depending on such variables as the costs of labor, transportation, space, advertising (donor recruitment) and consumables. Costs are not charges (Wallace, 2001). Blood collectors determine charges based on yet another set of variables that include specific blood group, whether the hospital is a favored 'sole source' customer or a supplemental user, how the particular center derives its charges (which may depend on how it charges for derivative components such as platelets and plasma), and whether the particular region is under competitive pressure for red cell pricing. Most collectors also charge additional fees for such services as gamma irradiation, filtration, washing and typing for specific antigens. An example of a Directory of Fees for red cells from a large regional blood collector is provided in Table 2.4. The costs that the transfusion service incurs for storage, handling, compatibility testing, issuance and infusion are added to the collector's charges, and the total is the charge that the hospitalized patient receives.

In 1992, an audit at Duke University Medical Center determined the base average direct and indirect hospital costs for providing a unit of red blood cells (Lubarsky *et al*., 1994). The Transfusion Service's base cost for providing an allogeneic unit of red blood cells was $113.58. That 'cost' to the hospital compares with the current $293 'charge' for red cells in Table 2.4. To obtain the actual hospital cost of transfusing a unit of red blood cells in the perioperative period, the investigators calculated associated costs, including compatibility tests on multiple units per each unit transfused in the perioperative period, performing ABO and Rh typing and antibody screening on samples from patients who were not subsequently transfused, compatibility tests on units not issued, handling costs of units issued but not used, physically administering the blood, and the cost of the recipient contracting an infectious disease or developing a transfusion reaction. These associated costs increased the cost of transfusing an allogeneic unit of red blood cells in the perioperative period by $37.62, or 33 per cent (Lubarsky *et al*., 1994).

Table 2.4 Fees for red cell preparations and special services, Greater Chesapeake and Potomac Region (July 2004–June 2005)

	$
Charges for components:	
Whole blood	373
Red blood cells	293
Leukoreduced red blood cells	308
Red blood cells, washed	460
Red blood cells, frozen, thawed, washed	619
Charges for special preparations:	
CMV negative	40
Irradiation	60
Antigen type, per antigen, per unit	70
Sickle negative	22
IgA negative	81
Import fee – rare unit	289
Unit search fee for rare unit	77

The implementation of (almost) universal leukoreduction, NAT testing, look-back and donor deferral policies, and increased donor screening have undoubtedly increased the cost of producing blood components since 1992 (Report to Congress, 2001). Also contributing to these increases are the rising costs of labor, regulatory compliance, voluntary industry standards, administration and overheads. In the US, fees charged to hospitals for each unit of red cells increased by over 8 per cent from 1999 through 2000, and the American Red Cross raised the price of red cells by 10–35 per cent in 2001 (Report to Congress, 2001).

The societal cost of an allogenic red cell transfusion in Canada has been determined using a cost-structure analysis and cost information from 2001 through 2002. Costs of blood collection, production, distribution, delivery (hospital transfusion service processing and patient administration), transfusion reaction management, and opportunity cost of donor's time were included in the analysis. Canadian Blood Services and Hema-Quebec supplied the data for collection, production and distribution stages. Eight hospitals across six Canadian provinces provided delivery and transfusion reaction costs. Inpatient costs were assessed for the intensive care unit, emergency, general medicine ward and operating room. The aggregate mean societal unit cost of red cells transfused to an inpatient in 2002

was 264.81 US dollars. The mean cost of blood collection, production, and distribution was 202.74 US dollars; the mean opportunity cost of donor time was 18.21 US dollars; the mean cost of hospital transfusion service processing was 16.65 US dollars, of red cell transfusion was 26.92 US dollars, and of transfusion reaction management was 0.29 US dollars. There were substantial variations in hospital transfusion service processing and RBC transfusion costs across hospitals. The societal unit cost of red cell transfusion was found to have doubled since 1995, and increases in unit costs would be expected to continue as additional safety measures are introduced (Amin *et al.*, 2004).

If the charge for a 'unit' of red cell substitute is to be compared with that of a unit of red cells, both collection and some transfusion service charges will have to be used. Charges for the uncomplicated postoperative red cell transfusion may be several hundred dollars. However, from Table 2.4 it is easy to see that for patients with multiple antibodies or those who require rare units, the charges may accrue rapidly until the cost per unit approaches $800–1000.

THE ROLE OF RED CELL SUBSTITUTES

From the standpoint of the clinician and the transfusion service, the ideal red cell substitute should deliver oxygen, cause few undesirable effects, require no compatibility testing, remain stable during prolonged storage, persist in the circulation, be easily reconstituted, and be available at a reasonable cost. From the standpoint of the patient, or better of the general public, any alternative to transfusion that would lower the risk of transfusion-transmitted infection would be worth a substantial cost increment. Most candidate red cell substitutes would undergo some pathogen inactivation treatment and nanofiltration process that would address this important issue. Candidate substitutes would also avoid the compatibility issues, the logistic problems with multiple blood types, and many of the problems of storing, transporting, and transfusing refrigerated cells that are at great risk for immune, mechanical and thermal lysis. The other risks of transfusion that might be avoided (fever, urticaria, TRALI, hypotension, anaphylaxis, immunomodulation) would have to be balanced against the potential toxicities of the candidate replacement. Although head-to-head comparisons are difficult, the question of immediate delivery and release of oxygen by stored red cells has long been an issue, particularly for the pediatric surgeon, and small molecules that deliver oxygen might address that concern.

Where might a red cell substitute be most valuable? Probably where blood is not immediately available. Trauma outside of the hospital, including in the military setting, comes to mind immediately. Refrigerated group O cells cannot be readily stored and transported on ambulances across the country. A similar application involves acute, unanticipated blood loss in surgery, and moderate blood loss during and after surgery, especially during periods of blood shortage or for patients who are difficult to transfuse. The product can be used as a bridge for patients with multiple antibodies or rare blood types until compatible blood can be located. An oxygen carrier can also bridge the period of potential hypoxic coma and death in patients with severe autoimmune hemolytic anemia until treatment effects remission of the disease (Mullon *et al.*, 2000).

The largest potential use involves 'blood sparing' or 'blood avoidance' during surgery – a primary study endpoint for some early clinical trials. For elective surgery, the potential exists to reduce an estimated 5 million units of perioperative red cells transfused annually in the United States. However, the short 12–48-hour half-life of these small molecules limits their blood-sparing utility. Mathematical modeling suggests that benefits will be confined primarily to non-anemic patients who undergo extreme hemodilution and sustain large perioperative blood losses (Brecher *et al.*, 1999). The potential applications (in North America) include an estimated 600 000 cardiac surgery patients, 625 000 orthopedic surgery patients, and 70 000 men undergoing radical prostatectomy. While the short half-life of current candidate substitutes all but precludes their use for managing chronic anemia, a 'second generation' product with a longer intravascular survival might find substantial application for the estimated 40 per cent of patients with lung or ovarian cancers who develop chemotherapy-induced anemia.

The largest potential market for oxygen carriers as red cell substitutes is in those parts of the world that can least afford to pay for it. In the developing world, 25 per cent of maternal deaths from pregnancy-related causes are associated

with loss of blood (WHO, 2001). Pediatric deaths from malaria-induced anemia are entirely preventable (English *et al.*, 2002). Operational problems, infrastructure costs and cultural issues pose seemingly insurmountable hurdles to building modern blood delivery systems in the same regions. Even if they could, a sizable fraction of the donor population carries infectious agents and donations would have to undergo a pathogen inactivation process. Inexpensive blood substitutes, even those that fail to meet the rigorous safety standards applied to blood in the developed world, would allow patients to receive treatments ordinarily requiring blood transfusion, and could improve health dramatically in many developing countries. Whether such a practice, or even clinical trials of such products, is ethically acceptable remains a subject of intense debate. If the problems of their high cost and short intravascular half-life can be solved, these drugs will save countless lives in the developing world.

EDITOR'S SUMMARY

Enormous strides have been made in assuring the safety of the blood supply since HIV was recognized, in the early 1980s, as being transmitted by blood transfusion. Nevertheless, the supply is tenuous, with the average age of the population increasing in the developed world, and older patients receiving a disproportionate amount of the blood transfused. Furthermore, costs of transfused blood continue to rise as new tests are introduced and the complexity of regulatory compliance and labor costs increase.

The impact of a blood substitute in the developed world would initially be in cases where red blood cells are not immediately available, such as in certain trauma cases and in patients for whom alloimmunization precludes location of adequately cross-matched blood. However, blood substitutes could offer further advantages in added safety in regard to infection risk and more efficient delivery of oxygen to tissue. The potential for benefit in the developing countries is especially promising, as many of these countries lack the sophisticated blood banking systems of the developed world, not to mention a reduced number of qualified donors.

The full impact of a red blood substitute cannot be fully assessed until one or more products are available for clinical use, because any benefit will be balanced against any shortcomings such as side effects and limited intravascular persistence.

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Chapter 3

Regulatory Perspectives on Clinical Trials for Oxygen Therapeutics in Trauma and Transfusion Practice

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GENERAL REGULATORY BACKGROUND IN THE UNITED STATES

In the United States, new drugs are approved under authority of section 505 of the Federal Food, Drug, and Cosmetic Act (United States Code, Title 21, Chapter 9; *Guidance for Industry*, 1998). Section 505 of the Act (21 U.S.C. §355) describes the legal framework for the evaluation, review, and approval of new drugs for introduction into interstate commerce. Under section 505(a), 'no person shall introduce or deliver into interstate commerce any new drug, unless an approval of an application filed … is effective with respect to such drug'. Following passage of the Food, Drug, and Cosmetic Act in 1938, drug manufacturers were required to show only that a candidate drug was safe. In 1962, partly in response to the thalidomide tragedy, the United States Congress amended the Federal Food, Drug, and Cosmetic Act to require that in addition to safety, effectiveness had to be demonstrated in order to support approval to market drug and biologic products in the United States. These amendments included provisions that required manufacturers of drug products to provide 'substantial evidence' of a drug's effectiveness. 'Substantial evidence' is defined in section 505(d) of the Act as 'evidence consisting of adequate and well-controlled investigations, including clinical investigations, by experts qualified by scientific training and experience to evaluate the effectiveness of the drug involved, on the basis of which it could fairly and responsibly be concluded by such experts that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the labeling or proposed labeling thereof'.

Since 1962, there have been many discussions about the meaning of the term 'substantial evidence' and the quantity and quality of the evidence needed to demonstrate effectiveness. It had been FDA's position that Congress intended to require at least two adequate and well-controlled studies, each convincing in its own right, to establish effectiveness. In section 115(a) of the 1997 Food and Drug Administration Modernization Act (FDAMA) (the Modernization Act) for human drug and biological products (Public Law 105–115), Congress amended section 505(d) of the Act to clarify that the Agency may consider 'data from one adequate and well-controlled clinical investigation and confirmatory evidence' to constitute substantial evidence if FDA determines that the data and evidence are sufficient to establish effectiveness (*Guidance for Industry*, 1998).

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Biological products are approved under authority of section 351 of the Public Health Service Act (PHS Act) (42 U.S.C. §262). The requirements for licensure include a showing that the products are 'safe, pure, and potent'. 21 CFR 600.3(s) states, 'The word potency is interpreted to mean the specific ability or capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended, to effect a given result.' Starting in 1972, FDA began a process for reviewing the safety and effectiveness of all previously licensed biologics and stated that in this review process, proof of effectiveness would consist of controlled clinical investigations as defined in the provision for 'adequate and well-controlled clinical studies' for new drugs (21 CFR 314.126) unless waived as not applicable to the biological product or essential to the validity of the study and that an alternative method is adequate to substantiate effectiveness (21 CFR 601.25(d)(2)). Serological response data have been identified as examples of adequate alternatives 'provided that a previously accepted correlation with clinical effectiveness existed' (Guidance for Industry, 1998).

In 1998, the United States Food and Drug Administration (FDA) published *Guidance for Industry: Providing Clinical Evidence of Effectiveness for Human Drug and Biological Products*. The document was intended 'to provide guidance to applicants planning to file new drug applications (NDAs), biologics license applications (BLAs), or applications for supplemental indications on the evidence to be provided to demonstrate effectiveness'. In addition, the document was intended to meet certain other requirements of FDAMA. Although the evidentiary standard for biological products was not changed by the 1997 amendment to the law, Congress directed the agency to take measures to 'minimize differences in the review and approval' of products required to have approved Biologics License Applications (BLAs) under section 351 of the PHS Act and products required to have approved NDAs under section 505(b)(1) of the FDC Act. This guidance document, which may be found on the FDA website at http://www.fda.gov/cber/guidelines.htm, discusses in detail the scientific reasoning underlying the legal standard for demonstration of substantial evidence of clinical effectiveness and describes the rationale for both the quantity and quality of evidence needed to support effectiveness.

In order to meet regulatory requirements in the United States, any clinical trial in support of an NDA designed to meet the standard of demonstrating 'substantial evidence of effectiveness' must also meet the requirements for being 'adequate and well controlled' as defined in the Code of Federal Regulations at 21 CFR 314.126. In order for clinical trial results to meet the regulatory requirements, there should, among other things, be a clear statement of the objectives of the investigation, a summary of the actual methods of analysis in the study protocol and in the report of the results of the study. Claims being sought should be defined prospectively, i.e., before the study begins, and they should be amenable to evaluation using outcomes that are direct measures of clinical benefit. The design should permit valid comparison with a control so that a quantitative assessment of the treatment effect can be made.

On occasion, an applicant can use a surrogate marker of efficacy (surrogate-endpoint), as for example under the provisions for accelerated approval (21 CFR 601.41 or 21 CFR 314.510) which state that 'FDA may grant marketing approval for a new drug on the basis of adequate and well controlled clinical trials establishing that the drug product has an effect on a surrogate endpoint that is reasonably likely, based on epidemiologic, therapeutic, pathophysiologic, or other evidence, to predict clinical benefit. …' One definition in the literature states that a surrogate marker is a laboratory measurement or physical sign used as a substitute for a clinically meaningful endpoint that measures directly how a patient feels, functions or survives (Meinert, 1986; Temple, 1995). Generally, an effect on a valid surrogate marker would correlate with a meaningful clinical outcome (Temple, 1995; Friedman *et al.*, 1996). If the pathophysiology of a disease and/or the mechanism of action of a therapeutic intervention are well understood, it may be possible to link specific effects on the surrogate marker to anticipated effects on a clinical endpoint of interest. A valid surrogate marker has biological plausibility; the effects of an intervention on the surrogate should predict the effects of the intervention on a clinical outcome. It is important to note that a surrogate endpoint might be valid for one clinical effect but may have no relationship to another. It is also important to keep in mind that the relationship between a surrogate marker and the clinical event of interest may not be causal; the relationship may

be coincidental or the surrogate and the clinical event of interest may both be independently related to some third factor (Temple, 1995; Friedman *et al.*, 1996).

For safety purposes, clinical trials capture information about new and/or novel adverse events, as well as quantitative and/or qualitative increases in expected adverse events above their underlying background rate/intensity. Studies may also be designed to capture interactions of study product with a wide variety of co-morbid conditions. As noted above, for any indication, a drug or biologic must have a favorable benefit to risk profile.

CONSIDERATIONS FOR CLINICAL TRIALS IN TRAUMA

Many papers have been written about the pathophysiology of hemorrhagic shock and trauma, and medical interventions that might improve the clinical outcome following trauma (Moore *et al.*, 2004). There have been a number of randomized clinical studies in this population to evaluate whether a given intervention improves outcome; a number of these trials have demonstrated lack of effectiveness or resulted in negative safety findings with regard to the specific intervention(s) under investigation (Sloan *et al.*, 1999; Riou *et al.*, 2001). Clearly, more research is needed in order to elucidate the pathophysiology of trauma and to discover what endpoints are most appropriate for clinical trials.

In an attempt to identify safety and efficacy criteria of oxygen therapeutics in the treatment of hemorrhagic shock and trauma that would address the regulatory requirements discussed above, FDA's Center for Biologics Evaluation and Research (CBER), the National Heart, Lung, and Blood Institute, the Department of Defense, US Army Medical and Material Command, and the Armed Services Blood Program Office sponsored a workshop in 1999 entitled 'Criteria for Safety and Efficacy Evaluation of Oxygen Therapeutics as Red Cell Substitutes', held on September 27–28, 1999. The transcript of this workshop is available on the CBER web page at www.fda.gov/cber/minutes/workshop-min.htm.

In trauma, mortality is an unambiguous endpoint that many consider to be the most meaningful of clinical outcomes. Although short-term (e.g. 24–48 hours) survival is helpful in assessing the physiologic activity of a drug such as an oxygen therapeutic, long-term survival is the primary clinical benefit of interest to the patient and the patient's family. In other words, the benefit of short-term survival is limited if it does not lead to long-term survival. At present, there is insufficient information to correlate these two outcomes for, for example, oxygen therapeutics.

Recently, suggestions have been made about the use of composite endpoints that include major morbidities together with death, in an attempt to reduce the sample size needed to evaluate new therapeutic interventions in trauma (Moore *et al.*, 2004). Unfortunately, incomplete understanding of the pathophysiology of trauma and its attendant complications, and disagreement over the relative weights to be assigned to each component of the composite, have made this approach difficult.

Historically, many researchers have considered field use as the most likely situation in which oxygen therapeutics could improve survival. However, in major urban areas with rapid transit to definitive care, there may only be a small percentage of patients for whom use of an oxygen therapeutic might provide a survival benefit. Some of the most seriously injured patients will die in spite of rapid availability of optimum definitive care and other, less seriously injured, patients will survive with current resuscitation measures. Identifying those patients most likely to benefit from a therapeutic intervention, such as an oxygen therapeutic in the pre-hospital setting, has been very difficult because it requires assessments that may not be possible in the field or within the timeframe for making decisions about the therapeutic intervention (Moore *et al.*, 2004). It is therefore difficult to select deliberately and prospectively the small population of trauma patients for whom use of an oxygen therapeutic may provide a survival advantage. If a clinical trial is not designed to select deliberately and prospectively for this small subset of patients who are likely to benefit from treatment, generally large numbers of patients may need to be enrolled in order to test for a statistically significant survival advantage (Moore *et al.*, 2004). Given these considerations, as noted above, many more subjects could receive an oxygen therapeutic agent than are likely to benefit from such use. In addition, for trauma patients who have also sustained head injuries, the heterogeneity in the severity of head injury may lead to mortality outcomes independent of the effect of blood loss or the use of an oxygen therapeutic agent.

In rural areas or other situations where there may be prolonged delay to definitive care, there may be greater potential for an oxygen therapeutic to provide clinical benefit than in urban settings. Such studies are difficult to control and may pose complications of trial design and data analysis, due, for example, to practical considerations such as differences in the length of time required to transport a patient to the hospital. Nevertheless, in the rural setting, a temporary treatment that sustains adequate tissue oxygenation and aerobic metabolism prior to control of bleeding and/or prior to obtaining cross-matched blood may provide a clinical benefit.

In considering trials in the trauma setting, it is necessary also to think about issues of consent. On 2 October 1996, FDA published notice in the Federal Register (61 FR 51497) modifications to regulations governing informed consent under 21 CFR Parts 50, 56, 312, 314, 601, 812, and 814 (Federal Register documents are available through http://www.fda.gov). This publication followed significant public discussion of a draft rule published in September 21, 1995 at 60 FR 49086. The intent of the regulation was to provide 'a narrow exception to the requirement for obtaining and documenting informed consent from each human subject, or his or her legally authorized representative, prior to initiation of an experimental intervention'. The exception was intended to apply to a limited class of patients who were in need of emergency intervention but who could not give informed consent because of the life-threatening nature of their condition, and for whom legally authorized representatives could not be rapidly located. The principles discussed in the proposed rule were based on, among other things, the Belmont Report (available at http://www.hhs.gov/ohrp/humansubjects/guidance/belmont.htm), and encompassed the ideas of respect for persons, beneficence, and justice. Briefly, the preamble to the proposed rule stated that 'In emergent situations, protection (of the subject) is provided and the principle of respect for persons is satisfied if, in circumstances of clinical equipoise, either the test therapy or its historic alternative is provided, even without consent. When the relative benefits and risks of the proposed intervention, as compared to standard therapy, are unknown, or thought to be equivalent or better, there is clinical equipoise between historic intervention and the proposed intervention …'.

21 CFR 50.24(a) notes that the institutional review board responsible for the review, approval, and continuing review of the clinical investigation must document certain features in order to allow to proceed a trial under exception from informed consent requirements. 21 CFR 50.24(a)(1) and (a)(2) note that 'the human subjects are in a life-threatening situation, available treatments are unproven or unsatisfactory …' and 'The subjects will not be able to give their informed consent as a result of their medical condition'. Comments on the proposed rule included discussions about the ethics of performing experiments on patients unable to provide consent, and whether the intent of the rule was to permit the 'study of potential improvements in the treatment of life-threatening conditions where current treatment is unproven or unsatisfactory, in order to improve interventions and patient outcomes'. 21 CFR Section 50.24(a)(3)(ii) requires that 'Appropriate animal and other preclinical studies have been conducted, and the information derived from those studies and related evidence support the potential for the intervention to provide a direct benefit to the individual subjects'. Section 50.24(a)(3)(iii) clarifies that an IRB, in considering whether to approve the conduct of the clinical study, must consider what is known about the medical condition or disease, what is known about current standard of care, and what is known about the proposed new intervention or treatment. In making its determination, the IRB must consider the risks of the proposed intervention and whether these risks are 'reasonable' in relation to all available information about both the standard of care, the disease/medical condition, and potential efficacy of the new treatment. The preamble to the rule notes that the 'criteria contained in the rule do not require the condition to be immediately life-threatening or to immediately result in death. Rather, the subjects must be in a life-threatening situation requiring intervention before consent from a legally authorized representative is feasible. Life-threatening includes diseases or conditions where the likelihood of death is high unless the course of the disease or condition is interrupted.' Thus, people who might survive for long periods might be included in such studies even though the likelihood of survival is not known during the therapeutic window of treatment. In the preamble to the final rule (61 FR 51509), FDA noted examples of conditions where this situation might pertain. 'People with the conditions cited in the examples provided in the comments – e.g. long-term or permanent coma, stroke, and head injury – may survive for long periods, but the likelihood of survival is not known during the therapeutic window of treatment. People

with these conditions are clearly at increased risk of death due to infection, pulmonary embolism …'.

Section 50.24(a)(4) states that exception from informed consent requirements for emergency research may be granted if 'The clinical investigation could not practicably be carried out without the waiver'. This provision in the regulations engendered significant discussion about the meaning of the word 'practicably'. One comment on the proposed rule stated that 'practicability' should not refer to convenience, cost, or speed. FDA commented that the provision in the regulation was intended to recognize that 'within the realm of ethically proper actions (there is) a hierarchy of values and that (one) should seek the highest level of those values feasible in this situation'. FDA has understood that the 'principle of respect for persons of diminished autonomy applies in such a way that the less autonomy a subject possesses, the less suitable that subject is for research even if the research shows promise'. Therefore, 'if scientifically sound research can be practicably carried out using only consenting subjects (directly, or in most cases for the research contemplated in the rule, with legally authorized representatives), then the agency thinks it should be carried out without involving non-consenting subjects'. If results from a clinical trial performed in subjects who are able to give consent can be generalized to subjects who cannot give consent, then the study would not meet the criteria set out in the 21 CFR 50.24 (61 FR 51513).

Community consultation is one component of additional protections of the 'rights and welfare of the subjects …'. Section 50.24(a)(7) requires 'consultation (including, where appropriate, consultation carried out by the IRB) with representatives of the communities in which the clinical investigation will be conducted and from which the subjects will be drawn'. Section 50.24(a)(7)(iii) also requires public disclosure, 'prior to initiation of the clinical investigation, of plans for the investigation and its risks and expected benefits', and 'of sufficient information following completion of the clinical investigation to apprise the community and researchers of the study, including the demographic characteristics of the research population, and its results …'.

Section 50.24(b) notes that the 'IRB is responsible for ensuring that procedures are in place to inform, at the earliest feasible opportunity, each subject, or if the subject remains incapacitated, a legally authorized representative of the subject, or if such representative is not reasonably available, a family member, of the subject's inclusion in the clinical investigation, the details of the investigation, and other information contained in the informed consent document'. The IRB is also responsible for ensuring that the subject or legally authorized representative knows that (s)he may discontinue participation in the study at any time without penalty.

It is clear from this discussion that studies to be performed under the provisions of exception from informed consent require careful thought and consideration to accepted ethical precepts. As discussed in the preamble to the proposed rule, the Belmont Report anticipated that 'these (ethical) principles cannot always be applied so as to resolve beyond dispute particular ethical problems. The objective is to provide an analytical framework that will guide the resolution of ethical problems arising from research involving human subjects … The Belmont Report did not, therefore, address resolution of conflicts among these (justice, autonomy, beneficence) ethical principles that might be occasioned by a particular research protocol, but it did provide a framework within which conflicts among the principles could be resolved.' Unless an IRB responsible for the review, approval, and continuing review of the clinical investigation finds that the therapeutic intervention holds out the prospect of a direct benefit to subjects being treated in the study (21 CFR 50.24(a)(3), it would not be possible to conduct such a study under exception from informed consent.

CONSIDERATIONS IN TRANSFUSION AVOIDANCE

Red blood cells are given to prevent or treat significant, symptomatic anemia. There has been extensive clinical experience with red blood cell transfusion resulting in a practical appreciation of relevant indications, benefits, and risks. There is also an extensive collection of data on red blood cells, the anemic state, and their interaction, resulting from years of basic and applied research (Carson *et al.*, 1996; Weiskopf *et al.*, 1998, 2000; Hebert *et al.*, 1999; Malone *et al.*, 2003). However, views about the relative risks of the anemic state and the benefits of transfusion are varied and there is considerable concern about transfusion-associated adverse events associated with immunosuppressive and microcirculatory effects of allogeneic red blood cells. Indeed, in critical care, some studies suggest

that a restrictive transfusion practice may be superior to a liberal transfusion strategy for most patients. One retrospective analysis of registry data of 15, 534 patients admitted during a three-year period to a single shock-trauma center reported that transfusion of red blood cells was a strong independent predictor of mortality when patients were stratified by measures of shock at presentation including serum lactate, base deficit, anemia, and shock index (heart rate/systolic blood pressure). Although transfusion was an independent predictor of outcome, a causal relationship between transfusion and outcome was not demonstrated conclusively; much additional research is needed to elucidate the mechanism(s) that account for the worsened outcome among transfused patients and caution must be exercised in interpreting these data to show that allogeneic red blood cell transfusion in and of itself results in worsened outcome (Malone *et al.*, 2003).

General guidelines for transfusion of red blood cells do not contain sufficient information for transfusion in specific clinical settings or in specific patient populations. The level of anemia that can be tolerated by a given patient depends on physiological status, age, and other co-morbid conditions such that easily applied generalizations for dosing of red blood cells cannot be made. Further, conditions for red blood cell storage are not perfect. During the storage period, red blood cells leak potassium and lose surface area due to budding of small microvesicles from the cell surface. Loss of membrane changes the shape of the stored red blood cell and increases cellular rigidity. Acidic conditions during storage result in depletion of 2, 3 DPG during the first 10 days of storage. As storage continues, cellular metabolism slows and cells begin to lyse. Thus, when to transfuse and under what conditions remain difficult questions for the field of transfusion medicine (Weiskopf *et al.*, 1998).

Because both infectious and non-infectious complications of transfusion occur infrequently, a clinical trial to assess with statistical significance the relative safety of oxygen therapeutics compared to red blood cells could require a very large sample size (Dzik, 2002). For therapeutic interventions that facilitate transfusion avoidance, reduction in the use of allogeneic blood can be a surrogate endpoint for reducing the complications associated with allogeneic blood. While the goal of any such intervention is, of course, complete avoidance of allogeneic transfusion, total avoidance of allogeneic blood in all patients is not likely to be possible. In a clinical trial, one possible endpoint could be avoidance of allogeneic transfusion in a pre-specified proportion of patients. Since all therapeutic interventions carry risks, the risks of an oxygen therapeutic intended to reduce or eliminate allogeneic red blood cell usage need to be compared to the risks of transfusing allogeneic blood to arrive at an overall risk/benefit evaluation (Buehler and Alayash, 2004). Safety is, therefore, a critical element in any evaluation of therapies or maneuvers used or performed with the intent of reducing/eliminating allogeneic blood usage. In this context, it is not clear whether merely delaying the time at which allogeneic red blood cells are administered without reducing total allogeneic exposure, would provide a benefit to the patient.

SUMMARY

Under provisions of the United States Food, Drug, and Cosmetic Act, FDA evaluates clinical trials of drugs and biologics for 'substantial evidence' of effectiveness. This statement has been understood to mean evidence consisting of adequate and well-controlled investigations by experts qualified by scientific training to evaluate the effectiveness of the drug, on the basis of which it could be concluded that the drug will have the effect it purports to have under the conditions of use in the labeling. The scientific underpinning for this requirement is based on random variability inherent in biologic systems that could result in a finding of efficacy from chance alone and/or from unanticipated/undetected systematic biases. Because of these considerations, FDA has generally asked for two adequate and well-controlled studies for approval purposes. On occasion, FDA may accept data from one adequate and well-controlled study if the results are statistically robust and compelling and the endpoint(s) includes mortality, irreversible morbidity, or prevention of disease with a potentially serious outcome.

For clinical trials in general, claims sought are defined prospectively, i.e., before performance of the study, and they are generally amenable to study using outcomes that are direct measures of clinical benefit. If a surrogate marker of efficacy is used in a clinical trial, the effect on the surrogate marker correlates with an equivalent effect on the clinical endpoint of interest. For safety purposes, clinical trials generally capture information about

new and/or novel adverse events, quantitative and/or qualitative increases in expected adverse events above their underlying background rate/intensity. Studies may also be designed to capture interactions of study product with a wide variety of co-morbid conditions. For any indication, a drug or biologic must have a favorable benefit-to-risk profile.

For clinical trials in trauma, mortality is an unambiguous endpoint and long-term survival is the clinical benefit of interest to the patient and the family. Surrogate markers that correlate with mortality should be evaluated and validated before being used in a clinical trial. For studies conducted under the provisions of 21 CFR 50.24, exception from informed consent requires the possibility of direct benefit to subjects enrolled in the clinical trial. The preamble to the rule suggests, and has been interpreted by FDA to mean, that the benefit be an improvement in survival.

With regard to transfusion and transfusion avoidance, general guidelines for transfusion of red blood cells do not currently provide sufficient guidance for transfusion in specific clinical settings. Research is ongoing to develop appropriate perioperative measures of adequacy of tissue/organ oxygenation. For products used as oxygen therapeutics ('blood substitutes'), investigators might consider attempting to develop and validate criteria for dosing individual products and to develop guidelines for dosing that are routinely available at the bedside. Because such criteria and guidelines are not currently available, FDA has accepted avoidance of allogeneic red blood cell transfusion as a surrogate for avoidance of adverse effects of transfusion. The surgical procedure studied optimally reflects the characteristics of the general surgical population, and the population enrolled in studies optimally reflects the clinical characteristics of the population likely to undergo the particular procedure. Because blinding is extraordinarily difficult, clinical protocols optimally incorporate strict transfusion/infusion criteria in order to minimize bias.

EDITOR'S SUMMARY

The regulatory approval process for new therapeutic involves demonstration of both the safety of the product and its efficacy. In the United States, these requirements are clearly specified in law. Blood transfusions are usually given in conditions of temporary emergency, such as after traumatic or surgical blood loss. Any clinical trial that would compare a blood substitute with no treatment in these circumstances would be unconscionable. Direct comparisons of the safety and efficacy of red blood cells and blood substitutes are extremely difficult to design, involving complex ethical problems of end point definition and informed consent.

Over the last decade it has seemed acceptable to demonstrate that the use of a new blood substitute product would reduce or eliminate the use of transfused (allogeneic) blood. Following this strategy for regulatory approval involves weighing the risks of red cell transfusion against the risks of infusion of a blood substitute. Since the US FDA first proposed that reduction of allogeneic blood might be an approvable endpoint, blood has become extremely safe, owing to the introduction of many new safeguards and tests used by modern blood banks. Side effects of blood substitutes such as mild hypertension, hematuria, minor dysphagia, flu-like symptoms or skin discoloration may have been acceptable given the risk of contracting AIDS from a blood transfusion in 1983, but this is not the case today. Furthermore, some of the Adverse Events found in recent Phase III clinical trials of both hemoglobin- and perfluorocarbon-based products have been much more worrisome, including stroke and death.

Assuming that industrial and academic research can solve the problems posed in clinical trials of current products, the pathway through to regulatory approval will have to be cleared. To date, no product has been approved for use as a substitute for red blood cells.

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SECTION

2

Physiological Basis

The goal of a 'blood substitute' is to deliver oxygen to tissues. Part 2 of this book reviews the basic mechanisms of oxygen transport in normal conditions, then considers the impact of an artificial oxygen carrier. It begins by considering the diffusive transfer of oxygen from red blood cells to tissue, including the effect of plasma hemoglobin and other oxygen carriers that are not confined to the red cell. These physical principles can be tested in artificial capillary systems, but in order to understand their *in vivo* effects the regulatory mechanisms of the microcirculation must be understood. Finally, all of the mechanisms must fit into general regulatory systems, called 'autoregulation'.

Chapter 4

Clinical Physiology: Oxygen Transport and the Transfusion Trigger

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INTRODUCTION

Without oxygen, there would be no animal life. Without a system to transport oxygen from the atmosphere to the sites of oxygen utilization, there would be no life more complex than the amoeba. If oxygen is not taken up from environmental air and presented to every cell of the body for more than a few minutes, metabolism becomes anaerobic, lactic acid is produced, and cell death will eventually follow. The oxygen transport system in the human body must be able rapidly to accommodate to increased oxygen demand (exercise), restricted oxygen supply (e.g. high altitudes) and a wide variety of disease states that may affect any link in the chain from lung to mitochondria.

Transfusions are given to restore disruptions in the oxygen transport system when a clinician believes that doing so will improve the supply of oxygen to tissue. When oxygen supply drops below a critical level, there is often relatively little time to diagnose the problem and correct it. Furthermore, very few interventions are available to the clinician: in some cases local blood flow can be restored, such as in coronary angioplasty or bypass surgery, but more often the only option is to transfuse red blood cells. Unfortunately, there is no clear marker of the imminent fall of oxygen delivery below a critical point, and the actual critical point may differ between organs or individual areas within organs. In the absence of such a marker, the clinician is usually left with using a surrogate, like hemoglobin concentration or hematocrit, as the basis for giving a transfusion. A unique and peculiar aspect of red cell transfusion is that the practice has never been demonstrated to be safe or effective in clinical trials, a stringent requirement for new therapeutic agents. Thus any new product must be compared against an undefined standard.

OXYGEN REQUIREMENTS

Oxygen is required for the efficient conversion of substrate to ATP, whose high-energy phosphate bond is subsequently converted into the power that drives all living processes (Figure 4.1). Without oxygen, ATP can still be produced by anaerobic metabolism, but the yield of ATP is much less and the product of such metabolism is lactic acid. When the supply of oxygen does not meet demand, the so-called 'anaerobic threshold' is crossed, after which an oxygen debt is incurred, lactic acid accumulates and, if oxygen is not resupplied, cells die. The concentration of oxygen within mitochondria that is required for aerobic metabolism is quite low, in the range of 2–3 mmHg, and under normal conditions oxygen

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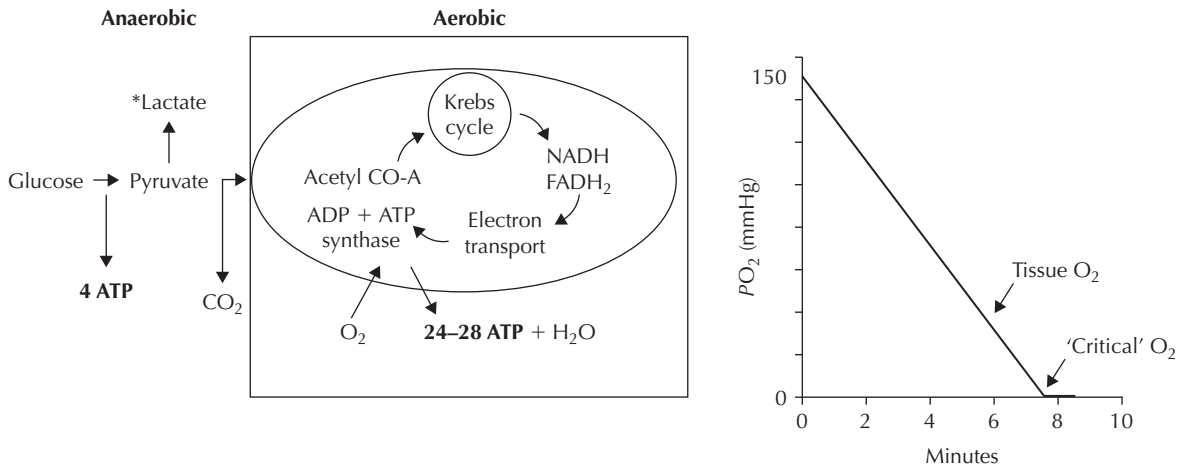

Figure 4.1 Metabolic oxygen consumption. With excess glucose and oxygen as substrates, mitochondria produce ATP for use in energy-consuming reactions (left). When oxygen supply to mitochondria is interrupted (right), PO_2 drops to critically low levels, the much less efficient anaerobic metabolism ensues, producing less ATP and lactic acid. If the oxygen supply is not re-established, oxygen debt, acidosis and tissue death follow. Mitochondria are able to utilize oxygen efficiently to very low levels, approximately 2–3 mmHg.

is in substantial excess, with tissue PO_2 in the range of 20–40 mmHg.

As these biochemical reactions evolved in an aqueous environment, oxygen is supplied to the smallest living creatures by simple diffusion. The rate of such diffusive oxygen transfer is described by the following equation:

$$\frac{\Delta O_2}{dt} = k_{O_2}\alpha_{O_2}\frac{\Delta PO_2}{dx} \qquad (4.1)$$

In this equation, k_{O_2} is the diffusion constant for oxygen, α_{O_2} is oxygen solubility, and ΔPO_2 is the difference in oxygen concentration from the source to the point of utilization. A very important point is that the solubilities of oxygen and carbon dioxide in aqueous solution (including plasma) are very low, approximately 0.02 ml/ml per atmosphere (Figure 4.2) (Winslow *et al.*, 1977), and the diffusion constant for oxygen is about 1.96 cm^2/s (Kreuzer, 1971). The constants for solubility and diffusion are slightly sensitive to temperature, solution viscosity and composition, but in general they are fixed within narrow limits. Thus any oxygen transport system that evolves beyond the single-cell stage must take these values into account.

In single-cell animals, the transport of oxygen from air to mitochondria is a diffusive process, and no circulation is needed. As organisms increase in size and complexity, the distances for diffusion are too great, and circulatory mechanisms are

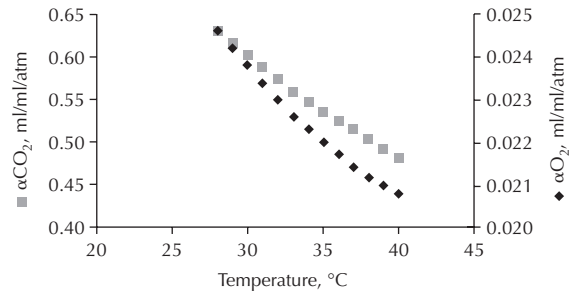

Figure 4.2 The solubility of the respiratory gases, oxygen and carbon dioxide, in aqueous solution. The values for plasma are shown, but the values for saline or water are very similar. Solubility for both gases is markedly temperature dependent.

needed to move oxygen from the lung to tissue sites of respiration. Oxygen delivery (DO_2) is the product of arterial oxygen content (CaO_2) and cardiac output (Q):

$$DO_2 = CaO_2 \times Q \qquad (4.2)$$

Oxygen consumption (VO_2) is the product of the arterial–venous oxygen difference ($C(a - v)O_2$) and cardiac output:

$$VO_2 = Q \times C(a - v)O_2 \qquad (4.3)$$

However in addition to the circulatory system, containment of an oxygen carrier (hemoglobin) within the red cell is also critical, as demonstrated by a simple calculation using Equation 4.3. If the

requirement for oxygen to support aerobic metabolism in a resting human is 5 ml/min per kg, and if the heart can pump 5 l/min, and if all of the arterial oxygen is extracted, then the minimum oxygen to be carried in arterial blood is (5 ml/min per kg × 70 kg)/(5 l/min) or 7 ml/dl of blood. The relationship between PO_2 and the concentration of oxygen in plasma is

$$O_2 = \frac{\alpha \times PO_2}{760} \qquad (4.4)$$

where α is the solubility coefficient in plasma (2.34 ml/dl per atmosphere). Thus, in order for plasma alone to present 7 ml/dl of blood to the capillaries, the PO_2 would have to be at least (7 ml/dl × 760)/2.34, or 2274 mmHg, or about 3 atmospheres – a clear impossibility. Furthermore, mammals such as man are capable of increasing their rate of oxygen utilization many-fold, reaching as high as 75–90 ml/min per kg in trained athletes. Support of oxygen transport in larger animals therefore presents two evolutionary challenges: first to transport large amounts of oxygen in blood such that it remains in the blood until it reaches respiring tissue, and second to provide mechanisms such that more blood flows to critical tissues in times of need.

The capacity to transport large amounts of oxygen is achieved by the presence of an oxygen carrier. Invertebrates transport oxygen in a circulating hemolymph that contains either hemocyanin (van Holde and van Bruggen, 1971), a copper-containing protein, or hemerythrin (Klotz, 1971), an iron-containing protein. In both of these, the metal atom is coordinated directly to the protein. However as body size increases still more, larger amounts of the oxygen carrier are required; but if the heme protein were free in the plasma it would turn over so fast that the larger organism could not possibly keep up with production. This problem is solved by the red blood cell, which packages hemoglobin in such a way that the molecules have a lifespan of approximately 100 days.

Red blood cells are uniquely suited to the task of oxygen transport for several important reasons. First, they contain a high concentration (about 35 g/dl) of hemoglobin, capable of carrying about 47 mL of O2 per 100 mL of red cells. Hemoglobin at this concentration could not circulate, but a suspension of cells in plasma where the cells occupy about 45 per cent of the volume of blood circulates very well. Thus, the overall oxygen capacity is about 18 ml/dl. Second, red blood cells are deformable; they can squeeze into capillaries that are smaller in width than the dimension of the cells themselves. This ensures a minimal distance for diffusion of oxygen from the alveolar space of the lung to hemoglobin, or from hemoglobin to sites of tissue respiration. Third, hemoglobin binds oxygen cooperatively. This means that very small changes in oxygen tension result in large amounts of oxygen either taken up in the lung or released in the tissues (Figure 4.3). Finally, hemoglobin demonstrates a Bohr effect: local conditions of pH and carbon dioxide affect the oxygen-binding behavior of hemoglobin in ways that are favorable for oxygen transport. Since the supply of oxygen is critical to organ function and therefore survival, it is expected that the physiologic mechanisms that ensure this supply are complex and redundant.

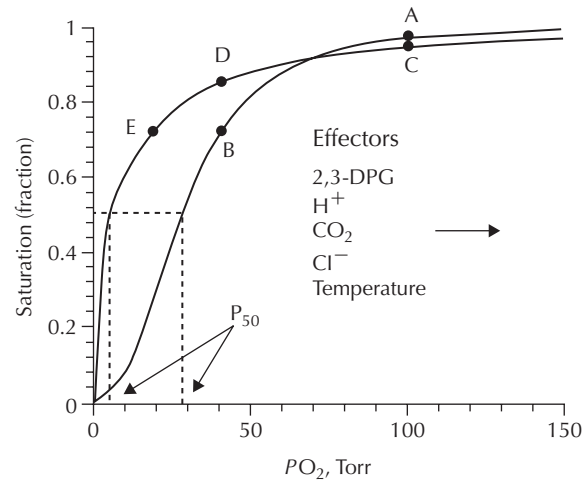

Figure 4.3 The normal whole-blood and myoglobin oxygen equilibrium curves. *P*50 is the PO_2 at which hemoglobin is half-saturated with oxygen. The principal effectors that alter the position and shape of the curve are indicated. Note that while both the hemoglobin and myoglobin curves allow saturation of about 95 per cent at normal P_AO_2 (A, C), hemoglobin unloads 23 per cent of its oxygen (B) and myoglobin only 7 per cent (D) at 40 mmHg. In order for myoglobin to deliver 23 per cent of bound oxygen, a venous PO_2 of about 16 mmHg would result in this example (point E).

LOCAL REGULATION OF OXYGEN SUPPLY: THE MICROCIRCULATION

In order to ensure adequate oxygen supply to tissues and to provide an oxygen reserve for

sudden increased demand, compensatory mechanisms must engage. This process, generally called 'autoregulation', is the process whereby the delivery of oxygen to tissues is matched to demand, and is discussed in depth in Chapter 9. The general mechanisms that can be altered to maintain tissue oxygenation are blood flow and oxygen extraction, and both have central nervous and peripheral components. Central mechanisms are comprised of the control of ventilation, hypoxic ventilatory responses, and carotid body reflexes and regulation of cardiac rate and stroke volume. Peripheral mechanisms are the result of local metabolic controls, and can be studied in denervated animal models. In such preparations when blood pressure is decreased, oxygen extraction increases. The oxygen extraction ratio (OER) is the fraction of oxygen that is removed from blood on a passage from the arterial to venous circulation, and is defined as:

$$OER = \frac{C(a-v)O_2}{CaO_2} \tag{4.5}$$

Arterial oxygen content (CaO_2), venous oxygen content (CvO_2) and their difference ($C(a-v)O_2$) are determined predominantly by the product of the hemoglobin concentration and fractional saturation, because normally approximately 97 per cent of blood oxygen is carried bound to hemoglobin, the remaining oxygen being physically dissolved in plasma.

As an example of an autoregulation experiment, Shephard *et al.* (1973) studied decapitated dogs, in whom blood pressure was maintained by infusion of epinephrine. When the blood pressure was lowered by either decreasing the epinephrine infusion or reducing the level of venous blood return to the heart, cardiac output fell precipitously to about 60 per cent of its control value. At the same time, the extraction of oxygen in the circulation increased by a comparable amount, so that tissue oxygen consumption (VO_2) was preserved. Thus the end result of autoregulation was maintenance of tissue homeostasis over a range of different conditions. This is a very important concept for transfusion practice, because it suggests that need for a one-to-one replacement of lost red cells is not necessary because of the healthy body's capacity to compensate through a variety of mechanisms. The problem comes in determining when the compensatory mechanisms are stretched to their limit in a given patient.

OXYGEN UPTAKE IN THE LUNG

In order to fully saturate hemoglobin as blood perfuses the lung, there must be adequate alveolar ventilation and perfusion. Because of the relatively large volume of 'dead' space (i.e., the trachea, bronchi and bronchioles) that is not capable of gas exchange, the PO_2 in the alveolus is significantly lower than the PO_2 in ambient air. For example, at sea level, where ambient PO_2 is about 150 mmHg, alveolar PO_2 is about 100 mmHg. The uptake of oxygen in pulmonary capillaries is a function of the gradient of PO_2 between mixed venous blood and alveolar PO_2. When flow (cardiac output) is normal, ample time is available for red cells to oxygenate as they transit pulmonary capillaries.

The diffusive uptake of oxygen in the lung is described by the diffusion equation:

$$\frac{d[O_2]}{dt} = \frac{100}{V_c} \times \frac{D_L}{60} \times (P_AO_2 - P_CO_2) \tag{4.6}$$

where $d[O_2]/dt$ is the rate of oxygen diffusion into the capillary, P_AO_2 is the alveolar PO_2, P_CO_2 is pulmonary end capillary PO_2, and D_L is the diffusion coefficient for oxygen in the lung and is regarded as a resistance to diffusion. V_c is the volume of capillary blood. Roughton and Forster (1957) described the components of this resistance as the in-series sum of a membrane component and the 'reactivity' of hemoglobin with oxygen:

$$\frac{1}{D_L} = \frac{1}{D_m} + \frac{1}{\theta_{O_2} \times V_c} \tag{4.7}$$

In this equation, D_m is the diffusion coefficient for the alveolar/capillary membrane interface and is proportional to the thickness of the membrane. θ_{O_2} is the reaction rate constant of oxygen with hemoglobin.

These relationships are conceptually simple, but their exact quantitation is complex. For example, θ_{O_2} is dependent on hemoglobin saturation, which in turn is dependent on the position of the oxygen–hemoglobin saturation curve (P_{50}), itself dependent on 2,3-DPG, pH, and PCO_2. pH and PCO_2 are further interdependent, and both are affected by the buffering of hemoglobin. Each of the terms in Equations 4.5 and 4.6 has a number of determinants, which can vary in different physiological conditions and in disease or pathological

states. While complex, all of these variables can be accounted for using mathematical models (Winslow, 1985). However, as a general conclusion, it is apparent that the optimal combination of the variables may be different between individuals, in different disease states, in different environments and in different settings of oxygen requirement.

THE 'OPTIMAL' HEMATOCRIT

What is the 'optimal' hematocrit? Obviously, there is no simple answer to this question and certainly none that would apply to all patients. Because it is easily measured, hematocrit is frequently taken as a surrogate for oxygen transport capability. The bulk viscosity of blood increases exponentially with hematocrit, and increased viscosity raises resistance to blood flow, limiting cardiac output in the absence of compensatory mechanisms. As the oxygen capacity of the blood (hemoglobin or hematocrit) increases, cardiac output decreases, and over a wide range of hematocrit there exists an optimum, defined as the point of maximal oxygen delivery. These principles have been studied theoretically (Crowell and Smith, 1967), in animals (Guyton *et al.*, 1973) and in humans with extensions to high altitude polycythemia (Winslow and Monge, 1987), and the general conclusion is that 35 per cent hematocrit represents the best combination of cardiac output and hematocrit in healthy animals and humans. Therefore, if all patients were in perfect health a transfusion trigger could be simply defined as 35 per cent.

The problem, of course, is that patients, by definition, are not in perfect health, and the ability to compensate for loss of hemoglobin by raising cardiac output, for example, may be quite variable. In addition, it is not always simple to determine which patients can utilize compensatory mechanisms and which cannot, or which ones are in greater danger of localized tissue ischemia because of restrictions such as coronary stenosis.

OXYGEN DELIVERY, OXYGEN UPTAKE AND THE 'CRITICAL OXYGEN'

A useful way to consider the limits of compensation was introduced by Cain and his co-workers (Cain, 1977). They compared the delivery of oxygen (DO_2, cardiac output × arterial oxygen content) with oxygen utilization (VO_2). This analysis led to the demonstration that as hematocrit is decreased (decreasing DO_2), there is no change in oxygen uptake until a 'critical' DO_2 is reached, at which point oxygen delivery to tissue can no longer be sustained (Figure 4.4). Thus, VO_2 is limited by oxygen demand above the critical DO_2 and limited by supply below the critical DO_2. Patients are in serious danger of organ failure if DO_2 is allowed to drop below the critical value, and the goal of transfusion therapy, is to maintain DO_2 well above that value so that an appropriate reserve of oxygen is maintained should the patient require it because of blood loss or elevated oxygen requirement.

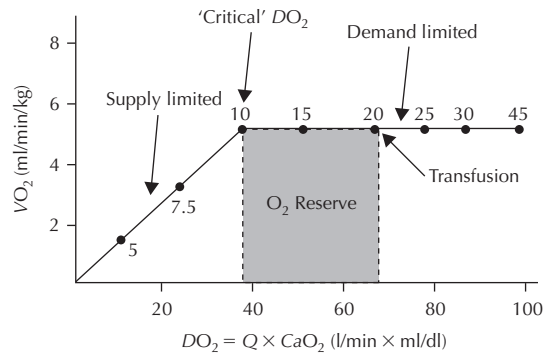

Figure 4.4 The critical DO_2. As the hematocrit is reduced in 5 per cent steps, VO_2 is maintained by a combination of rising cardiac output and falling PvO_2, resulting in a larger fractional oxygen extraction. When these compensations no longer suffice to satisfy tissue oxygen requirements, metabolic work cannot be maintained, and VO_2 falls resulting in an oxygen debt, rising lactic acid and deepening base deficit.

In summary, the capacity to regulate the supply of oxygen at a given level of arterial blood oxygen content is achieved by a combination of the ability of the heart to increase its output in response to increasing oxygen need, and the ability of the microcirculation to redirect blood flow to capillary networks by a system of vasoconstriction and vasodilation that operates at different levels of the circulation.

Viewed in this context of the evolution of oxygen transport systems, it is clear that the human system is complex and based on several critical elements including:

- low solubility of oxygen in plasma
- hemoglobin as the oxygen carrier

- red blood cells as the package for hemoglobin
- microcirculatory regulation of blood flow distribution.

The system of oxygen transport to tissues in humans is structured around these basic elements. Furthermore, the mechanisms that are in place to assure adequate delivery of oxygen to tissues have evolved in response to this system. Consequently, any physiologic strategy to satisfy tissue oxygen demands must function within the parameters of this system, and introduction of any solution that delivers oxygen in any way different from this system can be expected to trigger reactions that may not be predictable.

BLOOD TRANSFUSION AND OXYGEN SUPPLY

Acute blood loss (shock)

In considering the physiology of blood transfusion, it is useful to contemplate the changes that accompany blood loss. Acute blood loss can be considered at three levels: mild (up to 20 per cent blood volume), moderate (20–40 per cent blood volume) and severe (over 40 per cent blood volume). Most patients do not survive hemorrhage of greater than 50–60 per cent blood volume (Wisner and Holcroft, 1990). Compensatory mechanisms come into play in all three, but the degree of compensation varies with the severity of blood loss.

Apparently, chemoreceptors play little or no role in the acute response to blood loss at sea level. In environmental hypoxia, arterial PO_2 falls in spite of hyperventilation and alkalosis, and oxygen uptake in the lung is diffusion-limited. When cardiac output rises in this case, an increase in pulmonary red cell transit time will limit full oxygenation of red cells. Thus, the normal sea-level response to blood loss is mediated by baroreceptor (pressure) reflexes, including increased heart rate and ventilation. Both increase cardiac output, the former by increasing heart rate and the latter by increasing right heart filling. Hyperventilation causes a fall in $PaCO_2$ and rise in arterial pH, both of which increase arterial hemoglobin saturation via the Bohr effect.

The second response to acute hemorrhage, restoration of blood pressure, is mediated by the almost immediate secretion of vasoactive hormones, catecholamines and angiotensin II. The degree of hormone response is proportional to the degree of blood loss (Meyers *et al.*, 1991). Both mechanisms, baroreflex and hormone secretion, increase blood pressure and peripheral vascular resistance selectively – that is, blood flow is redistributed in a predictable way, decreasing sharply to muscle, skin, gut and kidney, while being preserved to heart and brain.

A third response to blood loss is a redistribution of water, from the extravascular to intravascular space. This redistribution is responsible for the falling hemoglobin concentration observed after hemorrhage, and occurs with impressive rapidity. Meyers *et al.* (1991) found in the fetal lamb that hemoglobin concentration was significantly lower after as little as 2 minutes following a 40 per cent controlled hemorrhage.

Several mechanisms account for this rapid refilling of the vascular space. First, falling hydrostatic pressure results in a net flux of water from the interstitial to intravascular space. Second, albumin is mobilized from the interstitial matrix and increases the oncotic pressure of the plasma. Third, albumin synthesis is increased in the liver, as is production of glucose and amino acids which further increase the plasma oncotic pressure. This increase in oncotic pressure contributes to refilling of the vascular space. Acute reduction of circulating blood volume can impair liver function (Ping Wang *et al.*, 1994).

As the vascular volume refills, hematocrit falls and, consequently, blood viscosity is lowered. Lowered blood viscosity reduces resistance to blood flow, increases venous return to the heart, and maintains or increases cardiac output (Guyton *et al.*, 1973). Reduced viscosity also reduces shear forces in the microcirculation and will lead to reduced release of local vasodilators (Kuchan and Frangos, 1993).

If these compensatory mechanisms are inadequate to preserve tissue oxygen requirements, lactic acid may be produced in ischemic tissues, dropping arterial pH. Acidosis presents a further stimulus to hyperventilation and reduces hemoglobin oxygen affinity via the Bohr effect. This may actually augment tissue oxygen delivery, especially in regions of the circulation in which oxygen requirements are high, such as in cardiac muscle or brain (Wasserman *et al.*, 1991).

Finally, erythropoiesis is stimulated. Although arterial PO_2 is preserved or increased in hemorrhage, the renal sensors which signal increased erythropoietin secretion must be sensitive to oxygen *flux* rather than oxygen

concentration (PO_2). In any case, erythropoietin concentration can be seen to rise within 2–3 days of hemorrhage, a reticulocyte response is present between 2–7 days, and, in humans, the hemoglobin concentration rises at about 7 days post-hemorrhage. Of course, in many surgical patients, this sequence of events may be delayed or prevented if there are other limitations to erythropoiesis, such as chronic inflammation, iron deficiency, renal failure or other concomitant problems.

Although most healthy patients can tolerate loss of up to 40 per cent of their blood volume, many surgical patients who are not healthy may be much less tolerant of blood loss. For example, elderly patients with ischemic heart disease may suffer tissue infarction as a result of decreased organ blood flow. Patients with borderline or inadequate pulmonary function may not be able to maintain arterial oxygenation when red cell capillary transit time decreases with increased cardiac output. Patients with liver disease may not be able to increase albumin synthesis to increase plasma oncotic pressure – and so on. These factors must be kept firmly in mind when considering the clinical indications for transfusion in an individual patient.

To some extent, restitution of blood volume by crystalloid and/or colloid resuscitation can in itself restore cardiac output. Thus, Mitzner *et al.* (1976) found that administration of epinephrine increased cardiac output by 55 per cent due partly to lowered right atrial pressure (caused by improved cardiac function) and increased pressure (caused by the volume shift to the systemic circulation). The choice of plasma expanders in the treatment of shock is still debated, but some workers believe that colloids such as urea-linked succinylated gelatin (Gelofusine®) and 6 per cent hetastarch (Hespan®) or Pentaspan® are more effective than crystalloid in restoring myocardial blood flow and oxygen after acute hemorrhage.

Chronic anemia

Transfusion practices in chronic anemia are not well documented in the literature, but clinicians tend to use the same hemoglobin and hematocrit 'triggers' as in acute anemia. This may not be appropriate since chronically anemic patients develop significant adaptation to their anemia, including increased cardiac output, expanded plasma volume and hyperventilation.

Transfusion in chronic anemia may be part of a disturbing indifference in physicians' attitudes and practices. Saxena *et al.* (1993) studied transfusions in 265 iron-deficient patients in a large metropolitan medical center, and found that in the majority of cases transfusions were given to raise the hematocrit or hemoglobin to arbitrary values, rather than using clinical signs or symptoms as a guide. Furthermore, iron was not prescribed for almost one-third of the patients, including those in whom blood was not given when it was clearly indicated.

Hemodilution

Much of the literature concerned with the transfusion trigger does not distinguish between acute blood loss and the more controlled condition, surgical hemodilution. The important difference is that in the latter instance, blood volume can be controlled in all but the most catastrophic cases. Thus, strategies for conservation of allogeneic blood in the surgical setting involve removal of blood prior to surgery and replacement with crystalloid or colloid (or both) so that during surgery loss of the patient's red cells can be minimized (Stehling and Zauder, 1991). These techniques can be very effective in blood-sparing and reducing a patient's transfusion exposure.

A separate but similar situation is the patient who presents with (relatively) longstanding and well-compensated anemia. One example of such patients is Jehovah's Witnesses who refuse transfusions on religious grounds (Marelli, 1994; Viele and Weiskopf, 1994; Polley *et al.*, 1998). Hemoglobins as low as 5 g/dl have been reported not to be associated with increased mortality. Similarly, the recommendation has been made that otherwise healthy African children with hemoglobins of 5 g/dl or higher not be transfused unless congestive heart failure is detected (Newton *et al.*, 1992). Some of these children can tolerate hemoglobin concentrations as low as 3 or 3.5 g/dl.

These examples from clinical practice illustrate the importance of understanding that if blood volume is restored, either in the hospital (hemodilution) or by physiologic refilling of the intravascular space (longstanding anemia), rather low hemoglobin concentrations can be managed successfully in many patients. This concept is in agreement with the argument that chemoreceptors are relatively less significant in adjusting to hemorrhage than are baroreceptors.

THE 'TRANSFUSION TRIGGER'

The 'transfusion trigger' is that event or set of events that results in a patient receiving a red cell transfusion. Excellent discussions have been published regarding the transfusion trigger (Levine *et al*., 1990; Goodnough *et al*., 1992; Stehling and Simon, 1994; Greenburg, 1995), and numerous conferences have been held to specify guidelines or algorithms whereby clinicians can make objective decisions about the use of red cells (Mollison, 1983; Consensus Conference, 1988; Robertie and Gravlee, 1990). For many years an empirical transfusion trigger was a hemoglobin concentration of 10 g/dl. If the value was less, the patient received at least two units of packed red cells or whole blood, in spite of the well-known fact that many patients tolerate modest anemia quite well (Mollison, 1983). The rationale for such transfusions was that an oxygen reserve needed to be maintained so that if unexpected (or expected) blood loss occurred during surgery, the patient would be in less danger of suffering deficient oxygen delivery to tissue. Guidelines for the transfusion of blood or packed red cells in the face of severe blood loss have been less well defined.

Historically, the risks of red cell transfusion were considered negligible. However, the HIV epidemic served to focus for the public, as well as for physicians, the risks of infectious disease transmission. The goal of 'zero risk' blood is now very close at hand (Schreiber *et al*., 1996), but the HIV experience has demonstrated the potential for further problems, and transfusion practices have changed permanently, with the implementation of effective measures for reducing patients' exposure to allogeneic blood.

Now that potential risks of blood products are focused more clearly, indications for all transfusions are carefully examined and transfusions must be justified in patients' records. While traditional transfusion triggers were based on hemoglobin concentration alone, the standard of care now dictates a more thorough evaluation of the need for transfusion, and the old '10 g/dl' rule is no longer adequate. Most physicians would agree that transfusion of males and females, young and old, and patients with ischemic coronary disease, for example, would not have the same triggers, but standards have not been available to sharply define individual differences. There are, in general, two types of indication for transfusion of red blood cells: inadequate oxygen delivery (anemia), and acute blood loss (volume depletion). They are different, and there is no reason, *a priori*, to assume the triggers should be the same in the two instances.

It is almost impossible to summarize the state of current transfusion practice, because data are very difficult to obtain. The European Sanguis study (Baele *et al*., 1994) found large variation from hospital to hospital in regard to the use of blood and blood products. Current experience suggests that otherwise healthy patients with hemoglobin values of 10 g/dl or greater rarely require perioperative transfusion, whereas those with hemoglobin values of less than 7 g/dl will frequently require transfusion (Consensus Conference, 1988). Further distinction between the needs of individual patients is not sharp. In the US, Goodnough *et al*. (1992) examined the hematocrits in respect to blood loss in a group of surgical patients and found 26 per cent of women but 13 per cent of men were transfused in excess of their estimated blood loss. In another study, Friedman *et al*. (1980) questioned whether the same 'trigger' should be used in both men and women, while the normal hematocrit range for the two sexes is well known to be different.

In our enthusiasm to spare patients the risks of blood transfusions, there is a risk of undertransfusion. While an NIH Consensus Conference (1988) recommended that the lower safe limit of hemoglobin concentration could be below 10 g/dl, no definite lower limit guideline was provided. Experimental studies by Wilkerson *et al*. (1988a) in hemodiluted baboons suggested that a hematocrit of 10 per cent could be reached before the oxygen extraction ratio rose or mortality increased. Cain (1977) showed that oxygen delivery was not critical until the hematocrit was less than 10 per cent in dogs. Levine *et al*. (1990) found adaptive physiologic changes during progressive hemodilution down to 15 per cent hematocrit, and a number of studies in Jehovah's Witness patients who refuse blood transfusion show that extremely low hemoglobins and hematocrits can be tolerated (Kitchens, 1993).

Animal studies underscore the risks of undertranfusion. Spahn *et al*. (1993) hemodiluted dogs in whom experimental stenoses had been placed on the left anterior descending coronary artery. They then measured regional function of the myocardium by sonomicrometry, and found that the lowest hemoglobin concentration tolerated without compromised function was 7.5 g/dl and an increase of as little as 1.9 g/dl by transfusion restored function and oxygen consumption in

the affected region. Other investigators have also found that coronary stenosis in dogs limits coronary oxygen supply reserve in progressive hemodilution (Levy *et al.*, 1993). In patients, a hematocrit lower than about 28 per cent is associated with significant cardiac ischemia (Christopherson *et al.*, 1991; Johnson *et al.*, 1992; Nelson *et al.*, 1993). These animal and clinical studies suggest that the current practice of tolerating hemoglobin concentrations as low as 8 g/dl or hematocrits of 28 per cent is probably as liberal as is reasonable, and that further lowering the hemoglobin 'transfusion trigger,' especially in patients at risk for coronary ischemia, could be very dangerous. In patients in whom the hematocrit must fall below 28 per cent, the astute clinician must assess the patient's ability to compensate for the reduced oxygen capacity and, if there is evidence of ischemia, careful monitoring of ECG, blood pressure, oxygen saturation and ST segment analysis is indispensable (Dick *et al.*, 1992).

Clinical transfusion triggers

Much has been written about the transfusion trigger since the NIH clinical conference in 1988 (Consensus Conference, 1988). In some ways, focusing attention on this issue has resulted in physicians' attitudes drifting away from using 'clinical judgment' toward the search for hard, quantitative triggers which relieve the clinician from the responsibility for making a decision (Faust, 1993). In reality, a decision to transfuse (or not to transfuse) is a clinical judgment, and the careful clinician distills many different data, objective and subjective, in coming to a final decision.

How then should the physician consider the decision to transfuse? Table 4.1 provides a general set of guidelines. Only in the rare instance where a patient is symptomatic should transfusion be considered when the hemoglobin is over 10 g/dl. The issue of the optimal hematocrit has been explored extensively in the literature, and it appears that there is little justification for maintaining a hematocrit over 35 per cent either at sea level or in high-altitude natives (Winslow and Monge, 1987), in view of the many compensatory mechanisms that can maintain DO_2 at or above this value (Guyton *et al.*, 1973).

When the hemoglobin is between 8 and 10 g/dl, the risk to most patients is very low. Some patients, especially elderly ones, will report subjective improvement in symptoms of shortness of breath or dyspnea on exertion when their hemaglobin is over 8 g/dl. Transfusion in these patients would appear to be justified, but elevation to values over 10 g/dl would seem unnecessary.

A hemoglobin concentration between 6 and 8 g/dl requires a thoughtful approach to the clinical evaluation of the patient. One should try to avoid transfusion, and alternatives are available such as lowering oxygen (e.g. rest, pharmacologic agents, hypothermia) or treatments to modify the cause of anemia (e.g. stop bleeding, treat underlying disease). However, if neither of these can be done, then a number of specific factors should be evaluated.

When the hemoglobin is less than 6 g/dl, few would argue with the decision to transfuse except when the anemia is of very longstanding. Such cases would include, for example, some patients with pernicious anemia who are well-adapted to

Table 4.1 Clinical transfusion triggers

Hemoglobin (g/dl)	Risk	Strategy
>10	Very low	Avoid transfusion
8–10	Low	Avoid transfusion if possible, but transfuse if demonstrably better after trial
6–8	Moderate	Try to avoid, decrease VO_2 *Clinical evaluation:* ● volume status ● pulmonary status ● cardiac status (ischemia) ● cerebrovascular status ● duration of anemia ● estimated blood loss during surgery ● extent of surgery and risk of rebleed
<6	High	Usually requires transfusion

a very low hematocrit. However, the adaptation is due, in part, to chronically increased cardiac output and expanded blood volume, and too vigorous transfusion can push the patient into overt congestive heart failure.

Physiological transfusion triggers

It should be possible to identify physiological transfusion triggers which might be more useful than hemoglobin or hematocrit. While no single trigger provides a clear-cut transfusion indication, the thoughtful clinician observes all possible signs of tissue ischemia and, based on experience, attempts to transfuse before the critical DO_2 is reached.

Hematocrit/hemoglobin concentration

The most commonly used parameter, the hematocrit (or hemoglobin concentration), is useful in some clinical situations but not in others, and it is important to be aware of its limitations. For example, in chronic anemia, an expanded plasma volume gives the impression that the red cell mass is smaller than it really is. In acute blood loss, some time is required for the fluid spaces to re-equilibrate and the hematocrit to once again reflect the red cell mass.

In one study of surgical patients, Cordts *et al*. (1992) found that while hematocrit significantly correlated with red cell mass both intra- and postoperatively, the ability of the hematocrit to predict red cell mass in individual patients was poor. In their study, hemodynamic parameters did not contribute to prediction of the red cell mass, plasma volume or total blood volume at any time, and the authors believe that some patients, particularly those at risk for coronary ischemia, may be undertransfused if traditional hematocrit 'triggers' are used. Similarly, Kim *et al*. (1993) found that the number of inpatient days did not correlate with the level of hemoglobin at discharge, preoperative hemoglobin, or drop in hemoglobin during hospitalization. They concluded that the use of blood transfusion as a means to shorten hospitalization is probably not justified. However, in another study of patients with postoperative myocardial ischemia (Nelson *et al*., 1993), a postoperative hematocrit less than 28 per cent was found to be associated with significantly more ischemic events than a hematocrit over 28 per cent.

The hemoglobin concentration in Jehovah's Witness patients who undergo surgery with severe anemia is only a predictor of overall outcome when less than 3 g/dl. Multiple independent factors influence outcome of these severely anemic surgical patients, the strongest being sepsis and active bleeding (Carson *et al*., 1988).

Mixed venous PO_2

Mixed venous PO_2 (PvO_2) would seem an obviously important parameter to assess adequacy of tissue oxygenation since, in theory, the mixed venous blood should be in equilibrium with tissue and its fall should indicate an increased OER. Some clinical studies have shown that PvO_2 decreases when oxygen is supply-dependent (see Figure 4.4) (Meyers *et al*., 1991). However, as is now known, the tissue PO_2 is much lower than mixed venous values (Tenney, 1974; Piiper *et al*., 1984) and PvO_2 can be normal even in severe anemia (Gould *et al*., 1983). Nevertheless, a decreasing PvO_2 has been used as a classic indicator of reduced tissue oxygenation, and perhaps a dropping value should be more meaningful to clinical evaluation of a given patient than an absolute value. Traditional textbooks of critical care medicine indicate that transfusions may be helpful when the PvO_2 drops.

Mixed venous oxygen saturation (SvO_2) may be a more useful indicator of severe oxygen extraction. Because of the steepness of the hemoglobin–oxygen dissociation curve, when mixed venous PO_2 falls below approximately 30 mmHg, the hemoglobin saturation falls rapidly. As the hemoglobin reserve depletes, small drops in PO_2 will depress the SO_2 more rapidly. SO_2 falls dramatically at hematocrits less than approximately 20 per cent. In one clinical study, Spiess *et al.* (1992) showed that SvO_2 is a sensitive indicator of overall VO_2 in liver transplant patients. In those patients, removal of the liver with attendant reduction in VO_2 produced a measurable rise in SO_2. When the new liver became functional, SvO_2 fell back to normal values.

Oxygen consumption (VO_2)

Hemoglobin concentration may limit maximal oxygen in some circumstances. However, the potential advantages of 'blood doping' have not been fully realized and, in general, the increased oxygen associated with transfusion has been marginal, and limited to well-trained athletes

performing maximal work (Turner *et al*., 1993). Extending these observations to patients may be of little value, since patients never consume oxygen at or near their maximal rate.

Published literature suggests that reduced VO_2 in postoperative and trauma patients is associated with a poor prognosis, and that increasing DO_2 by intervention (fluid boluses, administration of blood products, the use of inotropic drugs) reduces mortality (Yu *et al*., 1993). In contrast, other evidence indicates that the way in which DO_2 is increased is critical: volume resuscitation more effectively raises oxygen than transfusion, even though both raise DO_2.

In septic shock, VO_2 may be pathologically dependent on DO_2 (Cain, 1986; Biro *et al*., 1991; Slanetz *et al*., 1994). Patients whose cardiac output (and therefore DO_2) can be increased with dobutamine (Mink and Pollack, 1990; Lorente *et al*., 1993) or adrenaline (Seear *et al*., 1993) can increase VO_2, possibly by improving specific organ perfusion (Silverman and Tuma, 1992). However, when DO_2 is raised by increasing hemoglobin concentration by transfusion, no effect is seen on VO_2 (Lucking *et al*., 1990; Silverman and Tuma, 1992; Seear *et al*., 1993; Hanique *et al*., 1994). In fact, when 23 critically ill patients with sepsis were transfused with stored blood, not only did the VO_2 (measured by calorimetry) fail to rise, but there was also an inverse relationship between gastric mucosal pH and the age of the transfused blood (Marik and Sibbald, 1993), indicating poorer tissue oxygenation. In other studies, VO_2 failed to correlate with lactate levels in septic patients given fluid therapy, transfusions, or dobutamine (Conrad *et al*., 1990; Silverman, 1991; Steffes *et al*., 1991). These studies all indicate that pathological reduction of oxygen in sepsis is due to reduced tissue perfusion, not reduced oxygen content of the perfusing blood.

Acute respiratory distress syndrome (ARDS) patients may represent another case in which oxygen uptake and utilization do not agree: they may not increase VO_2 after increasing DO_2 (Ronco *et al*., 1990, 1991). Hanique *et al*. (1994) studied three groups of patients: those with sepsis, ARDS, and hepatic failure. They increased cardiac output by volume loading to increase DO_2, measured VO_2 by calorimetry and calculated VO_2 by Fick (Equation 4.3). They found that the calculated VO_2 (Fick) increased, while the measured VO_2 (calorimetry) did not. By using an increase in VO_2 as a criterion for successful transfusion, as many as 58 per cent of transfusions may be of questionable importance (Babineau *et al*., 1992).

Oxygen extraction ratio

The oxygen extraction ratio (OER) is the fraction of arterial oxygen delivery extracted by tissue (Equation 4.5). In dogs with experimental stenotic lesions in the left anterior descending coronary arteries, hearts did not raise their output in response to bleeding, showed greater lactate production, and failed at a higher hematocrit (17 per cent) than controls (10.6 per cent) (Levy *et al*., 1992). The authors of this study concluded that in the normal heart lactate production occurs when OER $>$ 50 per cent and hematocrit $<$ 10 per cent, but in the stenotic animals OER $>$ 50 per cent corresponded to a hematocrit $<$ 20 per cent. Thus, an OER $>$ 50 per cent indicated a need for transfusion, and the findings indicate that the transfusion trigger, in terms of hematocrit or hemoglobin concentration, is higher in hearts with underlying coronary ischemia. Similar results have been reported for primates (Wilkerson *et al*., 1988b), and the observations are consistent with experience with patients (Mathru *et al*., 1992).

Hemodynamic instability

In shock, reduced oxygen capacity and blood volume contraction exist at the same time. Most resuscitations are carried out first with volume expanders, then with replacement of lost red cells. However, it is not clear whether volume or oxygen capacity reduction is more important. Thus, Deitrich *et al*. (1990) studied patients with a variety of diagnoses who had undergone volume resuscitation. They concluded that an increase of DO_2 by transfused red blood cells did increase oxygen capacity, but they could not demonstrate any benefit measured as increased oxygen, decreased lactate or myocardial work. These observations lend support to the clinical dictum that in hemodynamic shock, raising the hemoglobin concentration must be accompanied or preceded by volume expansion.

Blood loss

Blood loss itself, apart from the attendant hemodynamic changes, is an important indicator of the need for transfusion. Carson and co-workers carried out a careful study of 125 surgical patients

who declined to be transfused on religious grounds and found that mortality was inversely related to hemoglobin concentration (Carson *et al.*, 1988). Mortality rose from 7.1 per cent for patients with hemoglobin concentrations over 10 g/dl to 61.5 per cent for those with hemoglobin concentrations below 6 g/dl. Mortality was 8 per cent for patients in whom 500 ml or less blood was lost, but rose to 42.9 per cent for those in whom loss was greater than 2000 ml.

Symptoms

Although a quantitative index of the need for a red blood cell transfusion would be optimal, in many instances, especially in cases of chronic anemia, the decision to transfuse is based on subjective symptom evaluation. None of the subjective symptoms is uniquely indicative of anemia *per se*, and so they must be evaluated in the context of the patient's total clinical presentation. However, these symptoms are of most benefit in patients in whom other more quantitative 'triggers' do not provide the clinician with a clear decision as to whether to transfuse. Unfortunately, many patients who require transfusions are unable to report symptoms because of their state of consciousness, or the use of sedatives or anesthetics.

Other signs of ischemia

It would seem that demonstration of hemoglobin-dependent oxygenation of specific tissues should be possible using modern sophisticated techniques of measurement. In general, this has not been the case. For example, when ^{19}F magnetic resonance spectroscopy was used to examine the *in vivo* bioenergetics in forearm muscles, no effect of increasing the hemoglobin concentration from 8.9 to 12.9 g/dl could be shown (Thompson *et al.*, 1992). Although oxygen is needed for wound healing, deposition of new collagen requires very little of it. Jonsson *et al.* (1991) were not able to demonstrate any correlation of hematocrit and collagen deposition in 33 postoperative surgical patients.

Individualized transfusion triggers

The oxygen transport system in higher organisms, such as humans, is the product of millennia of evolution. Since the supply of oxygen to tissue is critical for life, it should not be surprising that it is highly redundant and that there are many pathways for compensation when any of its components are compromised. The logical way to determine when a transfusion should be given is when clinical consequences of a reduced oxygen transport are established to be due to a deficit of red blood cells. This is not usually possible because of the many compensatory mechanisms that are engaged in anemia. Therefore, there is a long tradition of attempts to provide clinicians with guidelines in making a decision to transfuse. These are called 'transfusion triggers'.

The transfusion trigger may be not only an elusive goal, but also an inappropriate one. Physiologic studies cited in this chapter have shown animals can survive with hemoglobin concentrations lower than most clinicians would permit in patients. The NIH Clinical Conference (Consensus Conference, 1988) recommended that a 10 g/dl hemoglobin concentration might be too high for a transfusion trigger. When the hemoglobin is between 7 and 10 g/dl, many physiological and clinical data have to be considered before an intelligent decision can be made. Indeed, it might be time to abandon the concept of a 'transfusion trigger' in favor of a more sophisticated individual analysis of oxygen transport.

What effect should age, sex, heart disease, sickle cell anemia, or sepsis have on the decision to transfuse? The decision in an individual patient should be based on his or her need for augmented oxygen delivery to tissue, and the risk of not transfusing. Transfusion of blood or blood products is done for several reasons, and these should be carefully defined for each patient. The most important distinction that needs to be made is between the need for volume and the need for increased oxygen content of the arterial blood. The two do not necessarily go together: in acute hemorrhage, volume replacement may be more critical than it is in the chronically anemic patient in which oxygen content may be of primary concern.

There is no alternative to the exercise of good clinical judgment in the decision to transfuse a patient, but this requires an understanding of the fundamental determinants of oxygen transport and the way these determinants interact and compensate for anemia in individual patients. In approaching the decision to transfuse an individual patient, consideration must be given to each point in Table 4.1.

EDITOR'S SUMMARY

Transport of oxygen from ambient air to respiring tissue is critical to support life in all its variety, including health and disease, sea level and altitude, rest and extreme exertion. A fundamental physical restriction in this process is that the solubility of oxygen in plasma is so low that simple diffusion cannot satisfy tissue requirements. For this, a complex mechanism has evolved over eons, to include an oxygen carrier (hemoglobin), a system to protect hemoglobin from degradation and to protect tissue from toxic effects of exposure to iron (red blood cell), a pump (the heart), and a system that controls the distribution of blood to tissues according to some priority of need (the microcirculation). Each component of this system has its own capacity to compensate for alterations in other components, such that the entire ensemble works together in a finely coordinated manner. Most of the components of the oxygen transport system are fairly well understood; however, the last frontier is understanding how the circulation acts at the microscopic level to assure oxygenation of mitochondria. Techniques for the study of the microcirculation have only recently become available, and there are still important open questions. It should not be surprising that the introduction of a foreign or artificial replacement part into this picture (e.g., a 'blood substitute') would trigger unexpected reactions.

Transfusion is the practice of replacing one of the oxygen transport components: red blood cells. This is done when a clinician believes that the overall delivery of oxygen to tissue is (or is about to become) inadequate, placing tissue at risk. The factor(s) that lead to a transfusion being given are known as 'transfusion triggers'. There are many potential physiological markers to indicate a transfusion is required, but the essential problem with the transfusion trigger is that *the optimal transfusion is given before it is needed*, so the practical trigger remains a subjective, clinical one. It is hoped that as further research in the field of oxygen transport is done, a more precise picture of the status of tissue at risk of imminent death emerges. Only then will accurate transfusion triggers be implemented, allowing transfusions to be administered in a rational way.

The introduction of 'blood substitutes' will certainly impact any transfusion trigger, but until such products come into clinical use, the way their impact will be felt can only be a manner of conjecture. For example, most products under development have useful plasma effectiveness in the range of 1–2 days. Thus, in order to avoid a blood transfusion, they will either have to be administered repeatedly or else the marrow will have to regenerate itself in that period of time. A further complication is that the indications for 'blood substitute' use will likely overlap, but not be identical to, the indications for blood transfusion.

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Chapter 5

The Role of Oxygen and Hemoglobin Diffusion in Oxygen Transport by Cell-free Hemoglobins

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INTRODUCTION

Hemoglobin-based oxygen carriers have been under development for several decades for use in situations of blood loss and circulatory collapse. The primary stumbling block in the last few decades has been vasoconstriction, leading to capillary shutdown. Hemoglobin solutions with high oxygen-carrying capacity and an oxygen affinity similar to or lower than that of blood have long been reported to cause hypertension, reduced cardiac output and increased vascular resistance (Hess *et al.*, 1993; Ulatowski *et al.*, 1996; Migita *et al.*, 1997). *In vivo* microscopic effects of cell-free hemoglobins are now under investigation by direct measurements of arteriolar and venous diameters and PO_2 levels and functional capillary density (FCD) in the microcirculation of hamsters (Tsai *et al.*, 2004a, 2004b). FCD has been shown to be the primary correlating factor in survival in a shock hamster model (Kerger *et al.*, 1996, 1997). The molecular design of an effective hemoglobin-based oxygen carrier should be geared towards maintaining FCD, blood flow and oxygen delivery (Winslow, 2004).

In the 1980s, design of hemoglobin-based oxygen carriers was partly based on the assumption that the chemical oxygen-binding properties of the cell-free hemoglobin should mimic that of intraerythrocytic hemoglobin. It was believed that oxygen delivery, and thus efficacy as an oxygen carrier, could be maximized by lowering hemoglobin's oxygen affinity. For example, failed products such as diaspirin crosslinked hemoglobin (DCLHb) and recombinant hemoglobin (rHb1.1) were chemically or genetically modified, respectively, to display oxygen equilibrium curves similar to that of intact red blood cells in both the position (i.e., the *P*50) and shape (i.e., cooperativity) of the curves (Chatterjee *et al.*, 1986; Doherty *et al.*, 1998). Both of these products turned out to be highly vasoconstrictive and have been abandoned as clinical products. A polymerized hemoglobin product (Hemolink™) with oxygen affinity even lower than that of red blood cells has been shown to induce hypertension and was profoundly ineffective in prolonging survival in a rat hemorrhage model (Winslow *et al.*, 1998).

Strict compensatory mechanisms work to balance oxygen delivery to meet oxygen demand (Richmond *et al.*, 1999). These mechanisms alter vascular tone through either constriction or dilation to compensate for over or under delivery of oxygen, respectively, to keep oxygen levels within normal physiologic range (Guyton *et al.*, 1973). Observations in skeletal muscle microcirculation have shown a correlation between ambient PO_2 exposed to tissue, arteriolar diameter, and FCD;

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as PO_2 increases, arterioles constrict and FCD decreases (Lindbom *et al.*, 1980). In awake hamsters breathing 100 per cent oxygen, arterial blood PO_2 increased from 60 to 478 mmHg, which caused vasoconstriction in A1 to A3 arterioles, an increased systemic vascular resistance and a decline in FCD (Tsai *et al.*, 2003).

A fundamental flaw in the early design of cell-free hemoglobins was that differences in diffusive mechanisms of oxygen delivery by red blood cells and cell-free hemoglobins were not considered, even though it had been demonstrated in 1960 that the rate of oxygen transport was enhanced (i.e., 'facilitated') through cell-free hemoglobin solutions (Scholander, 1960). Based on the pioneering work on facilitated diffusion of oxygen by biologic oxygen carriers (Scholander, 1960; Wittenberg, 1966; Kreuzer, 1970), a theory was presented that cell-free hemoglobin-induced hypertension arises, at least in part, from an autoregulatory hemodynamic response to augmented oxygen transport, similar to that seen during hyperoxia (Vandegriff and Winslow, 1995; Winslow and Vandegriff, 1997). This theory challenged the assumption that cell-free oxygen carriers should be designed to have oxygen affinities similar to or lower than that of red blood cells. The physics underlying molecular diffusion had not yet been taken into consideration. The overall rate of oxygen transport can now be engineered to account for the diffusive properties of cell-free hemoglobin molecules and the facilitated diffusion of oxygen carried as oxyhemoglobin.

In 1927, Hartridge and Roughton showed that the rate of oxygen uptake by sheep red blood cells was lower by an order of magnitude than the rate measured for a cell-free hemoglobin solution at the same hemoglobin concentration. After confirmation of these results over several decades (see, for example, Roughton, 1959; Weingarden *et al.*, 1982), it was demonstrated that the lower rates of oxygen uptake and oxygen release by intact red cells compared to hemoglobin solutions are due to diffusional barriers surrounding red blood cells that are absent in hemoglobin solutions in either a rapid-mixing apparatus (Coin and Olson, 1979; Vandegriff and Olson, 1984a, 1984b) or in an artificial capillary with physiological relevant geometry (Lemon *et al.*, 1987). Later, enhanced rates of oxygen transport in the artificial capillary were also observed in mixtures of red blood cells and a cell-free hemoglobin (Page *et al.*, 1998).

OXYGEN DIFFUSION AND ITS BARRIERS IN THE CIRCULATION

The rate of oxygen transport by red blood cells is limited by diffusion of oxygen exiting the red cell through the intravascular layer of plasma between the red cell and tissue cells to its site of consumption at mitochondria (Hellums, 1977; Homer *et al.*, 1981; Federspiel and Popel, 1986). The intraluminal resistance to oxygen diffusion is comprised of several resistances acting in series:

1. The rate of oxygen diffusion inside the red blood cell is low because of the high intracellular hemoglobin concentration (35 g/dl hemoglobin) ($DO_2 = 2 \times 10^{-5}$ cm^2/s in water *versus* 0.75×10^{-5} cm^2/s inside RBCs; Kreuzer, 1970)
2. The solubility of oxygen (α) in plasma is low ($\alpha = 1.2074$ μM/mmHg; Winslow *et al.*, 1977)
3. The unstirred plasma layer surrounding red blood cells provides a diffusion barrier (Vandegriff and Olson, 1984b, 1984c)
4. The effective capillary surface area available for oxygen diffusion in a discrete particle model, depending on hematocrit, is about 50 per cent of the total capillary surface area (Hellums, 1977).

None of these diffusion barriers is present when hemoglobin is free in solution in plasma.

Physical chemistry of diffusion

According to the Second Law of Thermodynamics, molecules diffuse spontaneously in the direction of increasing entropy. When a molecular concentration gradient exists, the molecules will move until a new equilibrium is reached in which the molecules are distributed uniformly throughout the system. Diffusive flux is defined by Fick's Law of Diffusion to be proportional to the concentration gradient:

$$J = -D(\partial C/\partial x) \qquad 5.1$$

where J is diffusional flux, D is the proportionality (i.e., diffusion) constant, C is molecular concentration and x is the diffusion distance, such that $\partial C/\partial x$ gives the concentration gradient. Molecules in flux move with a drift velocity of D/kT, where D is the diffusion constant specific to the molecule, k is the Boltzman constant, and T is temperature in degrees Kelvin. As the molecules diffuse through the solution, they encounter a frictional resistance from the

solvent flowing around them. This expends energy, and the amount of energy expended is a function of the size and shape of the molecule and of the viscosity (η) of the solvent. According to Stoke's Law, for a sphere of radius r, the frictional resistance (f_0) is:

$$f_0 = 6\pi\eta r \qquad 5.2$$

Thus for a spherical molecule, its diffusion mobility is related to its size and the solution viscosity, according to the Stokes–Einstein equation (Atkins, 1978):

$$D = kT/6\pi\eta r \qquad 5.3$$

Oxygen diffusion *in vivo*

In vivo, oxygen moves by passive diffusion from the alveolar gas space in the lungs at high oxygen tension to the blood passing through the lungs at relatively lower oxygen tension. The chemical reaction binding oxygen to intracellular hemoglobin acts as a sink to load large amounts of oxygen onto hemoglobin carried as a new chemical species, oxyhemoglobin (HbO_2). Applying Fick's law to oxygenation in the lungs, the oxygen gradient is equal to the difference between the dissolved oxygen in the alveolar gas spaces ($PO_{2(lung)} \sim 100$ mmHg) and the mean pulmonary capillary oxygen concentration ($\alpha \times PO_{2(capillary)}$) in equilibrium with Hb-bound oxygen according to the oxygen affinity of the hemoglobin.

$$-J = DO_2[(\alpha PO_{2(lung)} - \alpha PO_{2(capillary)})/\Delta x] \qquad 5.4$$

Normal red blood cell hemoglobin is nearly fully saturated in the lungs. At normal red blood cell hemoglobin concentration of ~15 g/dl with 1.34 ml of oxygen bound per gram of hemoglobin, 20.1 ml of oxygen is bound to hemoglobin in 1 dl of blood. However, this calculation becomes invalid if hemoglobin oxygen affinity is too low to become saturated in the lung or if the red blood cell transit time is too rapid. In these cases, the concentration of free oxygen in plasma will rise, making the oxygen concentration gradient shallower and slowing the flux of oxygen into the lungs.

The diffusion gradient is in the opposite direction for oxygen release to tissues, as oxygen diffuses down its concentration gradient to its site of reduction at cytochrome oxidase. Since the oxygen concentration is zero where it is consumed, the gradient driving diffusion becomes directly proportional to the PO_2 in equilibrium with intravascular oxyhemoglobin, which again depends on the oxygen affinity of hemoglobin. Thus for deoxygenation, oxygen flux will be determined primarily by the vascular PO_2:

$$-J = DO_2\,[(\alpha PO_{2(vascular)} - \alpha PO_{2(mitochondria)})/\Delta x] \sim DO_2 \times \alpha PO_{2(vascular)}/\Delta x \qquad 5.5$$

Experimental results are consistent with this theory. The maximal rate of oxygen uptake (VO_2max) in muscle *in situ* is linearly related to mean capillary PO_2 measured as muscle venous oxygen tension (Hogan *et al.*, 1989).

For normal blood flow and red blood cell hemoglobin–oxygen affinity, the oxygen gradient in the lungs will be greater than in tissue – i.e., $\sim 100\,\text{mmHg} - PO_{2(capillary)} > PO_{2(vascular)} - 0$. Thus, oxygen efflux will be more dependent on the chemical reaction properties between hemoglobin and oxygen in peripheral tissues.

According to this simple analysis, hemoglobin solutions will enhance oxygen delivery to tissues by decreasing the diffusion distance, Δx; red cells flow down the center of vessels due to the Fåhraeus effect, while acellular hemoglobin molecules will distribute evenly throughout the vascular space by diffusion. In addition, the oxygen affinity of the hemoglobins will determine their degree of saturation in the lungs and their facility to offload oxygen at any given PO_2 in the peripheral vasculature.

FACILITATED DIFFUSION OF OXYGEN BY OXYHEMOGLOBIN

The process of selective transport across membranes facilitated by carriers is called facilitated transport (Way and Noble, 1992). In 1932, Roughton suggested that hemoglobin diffusion would add to the overall transport of oxygen within the red blood cell (Roughton, 1932). In the 1960s, *in vitro* studies of gases moving through hemoglobin solutions in membranes showed that nitrogen diffused in proportion to its pressure differential, whereas oxygen was transported by two processes:

1. Passive diffusion through the solvent in proportion to its pressure differential

2. An additive transport mediated by the hemoglobin solution in which the oxygen saturation was determined by the hemoglobin equilibrium curve (Scholander, 1960).

Hemoglobin-facilitated oxygen transport was determined to occur by the diffusion of oxyhemoglobin, so that a gradient of oxyhemoglobin is necessary for facilitated diffusion to occur (Wittenberg, 1966).

Facilitated transport of oxygen bound as a protein ligand serves as an extra term in the Fick equation, as defined in Equation 5.6 (Kreuzer, 1970; Wittenberg, 1970):

$$-J = \frac{DO_2\ \Delta[O_2]}{\Delta x} + \frac{D_{HbO_2}\ \Delta Y[Hb]_T}{\Delta x} \qquad 5.6$$

where D_{O_2} and D_{HbO_2} are the diffusion constants for oxygen O_2 and oxyhemoglobin, respectively, ΔO_2 is the change in oxygen concentration, such that $\Delta[O_2]/\Delta x$ gives the oxygen concentration gradient, ΔY is the fractional change in hemoglobin saturation, and $[Hb]_T$ is the total hemoglobin concentration, such that $([Hb]_T \times \Delta Y)/\Delta x$ gives the oxyhemoglobin concentration gradient. As long as oxygen is consumed outside the vessel wall, the diffusion distance, Δx, is the same for molecular oxygen and cell-free oxyhemoglobin – i.e., from the center of the vessel to its site of consumption. The difference, however, is that the flux for free oxygen is a linear function of the oxygen concentration, while the flux for oxyhemoglobin is a non-linear function of the oxyhemoglobin concentration gradient that is defined by the shape and position of the oxygen equilibrium curve (i.e., a property of the hemoglobin molecule).

According to the Stokes–Einstein relation (Equation 5.3), facilitated diffusion of oxygen is a function of the size of the oxyhemoglobin molecule and the viscosity of the hemoglobin solution. Wittenberg showed that oxygen flux through solutions of different sized hemoglobin molecules varied inversely with hemoglobin size; e.g., the hemoglobin–haptoglobin complex (117 kDa versus 64 kDa for human hemoglobin tetramer) decreased facilitated transport of oxygen by fivefold (Wittenberg, 1966). Scholander demonstrated the influence of viscosity by adding 10 per cent gelatin to the hemoglobin solution and measured the rates of transport of oxygen and nitrogen; the gelatin had a small effect on the passive diffusion of nitrogen, but decreased the facilitated component of oxygen diffusion by half (Scholander, 1960). Nishide *et al.* (1997) measured facilitated oxygen flux across a thin solution membrane as a function of hemoglobin concentration and solution viscosity, and found that flux was significantly decreased at higher hemoglobin concentrations in proportion with increasing viscosity of the solution.

For dual-mode transport across a membrane, i.e., simple diffusion facilitated by a carrier protein, the oxygen permeability coefficient, P, is defined as the product of its molecular diffusivity (DO_2) and solubility (α) plus a term for the oxygen transport via reversible oxygen binding to the carrier protein, e.g., hemoglobin:

$$P = \alpha DO_2 + \frac{[Hb]K_{eq}D_{Hb}}{1 + K_{eq}PO_2} \qquad 5.7$$

where K_{eq} is the equilibrium binding constant for the hemoglobin oxygen-binding reaction:

$$Hb + O_2 \rightleftharpoons HbO_2 \qquad 5.8$$

Using Equation 5.7, when P is plotted against $([Hb]K_{eq})/(1 + K_{eq} \times PO_2)$, the hemoglobin diffusion coefficient (D_{Hb}) can be estimated from the slope of the linear relationship; and αDO_2 is determined by the y-axis intercept.

Facilitated diffusion of cell-free hemoglobin

Data from an artificial capillary apparatus show the effect of facilitated diffusion on oxygen transport from an arteriolar-sized (~50 μm) microvessel (McCarthy *et al.*, 2001) (Figure 5.1).

Overall oxygen transport was measured by calculating the decrease in fractional saturation of the samples as they flowed down the artificial capillary against a flowing atmosphere of nitrogen surrounding the capillary. Two tetrameric hemoglobins were tested: unmodified HbA_0 with relatively high oxygen affinity (15 mmHg), and αα-Hb with oxygen affinity similar to that of red blood cells (33 mmHg) (see Figure 5.1). However, even though red blood cells and αα-Hb have similar $P50$ values (29 versus 33 mmHg) and Hill numbers (2.6 versus 2.4, respectively), the αα-Hb solution desaturated to a much greater extent compared to the red blood cell suspension, particularly at the longer residence times (see Figure 5.1, inset). Thus, the cell-free hemoglobin solution

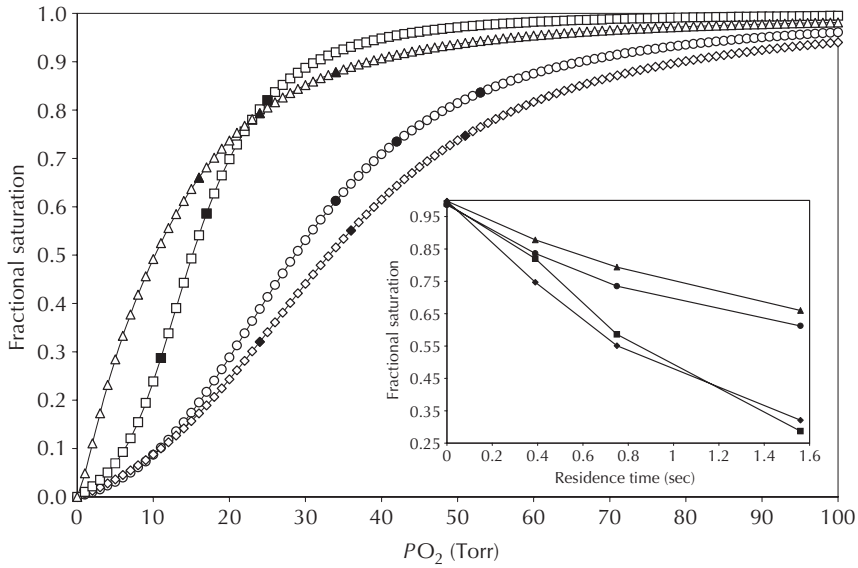

Figure 5.1 Oxygen equilibrium curves for red blood cells (circles), αα-Hb (diamonds), HbA_0 (squares) and PEG-Hb (BvHb-PEG5K 10) (triangles). The fractional saturation corresponding to each capillary exit PO_2 value at different flow rates is shown by the filled symbols. The inset shows the fractional saturation determined from the exit PO_2 as a function of capillary residence time. Reprinted from McCarthy *et al*. (2001), with permission from Elsevier.

displayed accelerated oxygen transport out of the capillary. Both tetrameric hemoglobins (αα-Hb and HbA_0) showed similar, highly desaturating profiles over time, even though their oxygen affinities were different. PEG-Hb, a polyethylene-glycol conjugated Hb (in this case, BvHb-PEG5K10, Enzon) showed less desaturation over time, similar to that of the red blood cells.

Reasons for the slowed rate of oxygen transport by the PEG-Hb solution compared to the tetrameric hemoglobin solutions are likely two-fold:

1. Calculations using the Stokes–Einstein equation (Equation 5.3) give a relative value for D_{HbO_2} for BvHb-PEG5K10 that is ~16-fold lower; the BvHb-PEG5K10 solution is more viscous (3.2 versus 0.9 cPs for the tetrameric hemoglobins; McCarthy, 1997) and the radius of the BvHbPEG5K10 molecule is larger (~14 versus 3 nm for the tetrameric hemoglobins; Vandegriff *et al*., 1997)
2. The oxygen equilibrium curves of the hemoglobins determine the amount of HbO_2 in equilibrium with the PO_2 of the solution (Figure 5.1). Based on the fractional saturation, the HbO_2 concentration gradient is dependent on how much oxygen will be offloaded from the hemoglobin at any given change in PO_2. The residence time in the capillary would have had to be increased to see the majority of the O_2 offloaded from the PEG-Hb.

At some longer residence time in the capillary, all hemoglobins would have become totally deoxygenated. And at infinite flow rate, none of the hemoglobins would have become desaturated because oxygen would not have had time to dissociate from the hemoglobin, and the rate of oxygen dissociation would have become limiting.

RATE LIMITING STEPS OF *IN VIVO* GAS EXCHANGE

Resistance of gas transfer in the pulmonary microcirculation was derived mathematically in the 1950s, giving the well-known linearized equation of Roughton and Forster (1957):

$$\frac{1}{D_L} = \frac{1}{D_M} + \frac{1}{\theta V_c} \qquad 5.9$$

D_L is defined as the overall diffusing capacity of the lung. The reciprocal of this value ($1/D_L$) gives the total resistance to gas movement in the lung (Forster, 1964). Thus, based on Equation 5.9, the overall resistance can be divided into two parts: diffusion of the gas across the pulmonary membrane into the plasma ($1/D_M$), and transfer of the gas from the plasma to intracellular hemoglobin (θV_c), where θ is the rate of gas uptake by red cells and V_c is the total volume of blood in the lung capillaries.

Rapid mixing techniques can be used to evaluate θ (see, for example, Hartridge and Roughton, 1927), and the data have shown that the rate of oxygen uptake by red cell suspensions (i.e., θ) is much lower than the rate of oxygen reaction with extracellular hemoglobin solutions. Further, it has been determined that hemoglobin chemical reaction rates are far too fast to be rate limiting *in vivo*, and equilibrium between hemoglobin and PO_2 can be assumed under normal conditions (Gibson *et al.*, 1955; Roughton and Forster, 1957). This assumption of equilibrium between hemoglobin and oxygen concentrations has been verified theoretically by Hellums and co-workers in the microcirculation (Baxley and Hellums, 1983; Hellums *et al.*, 1996). Therefore, it is the ratio of the chemical reaction constants, $K_{eq} = k'/k$, that is necessary to calculate oxygen transport by facilitated diffusion using the oxygen equilibrium curve.

However, it should also be considered that if either the association rate (k') or dissociation rate (k) was decreased drastically through protein mutation or modification, then the chemical reaction constant could become limiting and attenuate or eliminate the effect of facilitated HbO_2 diffusion. As an example, hemoglobin H and Ascaris hemoglobin have similar oxygen affinities, but the oxygen dissociation rate constant for Ascaris hemoglobin is one-thousandth that of hemoglobin H; hemoglobin H facilitates oxygen diffusion while Ascaris hemoglobin does not (Wittenberg, 1966).

For crosslinked tetrameric ($\alpha\alpha$-Hb) and a maleimide PEG-conjugated human hemoglobin (MP4, Hb-PEG5K6), the R-state oxygen affinities are dictated by the R-state dissociation rates because the R-state association rates are unaltered compared to native human hemoglobin (Vandegriff *et al.*, 1991, 2004). With these two modified hemoglobins, the R-state rates are faster compared to unmodified hemoglobin, even though $\alpha\alpha$-crosslinked hemoglobin has a $P50$ double of that of unmodified hemoglobin (33 versus 15 mmHg), while the PEG-conjugated hemoglobin has a much lower $P50$ (5 mmHg) and a primarily hyperbolic binding curve. Thus, regardless of the oxygen affinities of these two hemoglobins, their oxygen dissociation rates would not be rate-limiting for oxygen transport.

FACILITATED OXYGEN TRANSPORT AND VASOCONSTRICTION

The rates of oxygen transport in the artificial capillary apparatus described above (see Figure 5.1) showed a direct correlation with the mean arterial blood pressure in rats in response to exchange transfusion with the hemoglobins studied (McCarthy *et al.*, 2001). Both $\alpha\alpha$-Hb and HbA_0, which gave higher calculated values for the diffusion transport parameter compared to RBCs and PEG-Hb, exhibited hypertension in the rat model, while the PEG-Hb did not.

The autoregulatory theory of hemoglobin-induced vasoconstriction predicts that the pressor effect can be reduced or eliminated by controlling the rate of oxygen transport by the cell-free hemoglobin. If the rate of oxygen transport is similar to that of red blood cells, then compensatory mechanisms will respond normally to changes in oxygen delivery. From Equation 5.6, the overall flux of oxygen can be controlled by changing the oxyhemoglobin diffusion constant (D_{HbO_2}) and/or the hemoglobin saturation gradient, $\Delta Y[Hb]/dx$. As described by the Stokes–Einstein equation (Equation 5.3), the macromolecular diffusion constant (D_{HbO_2}) is inversely proportional to the viscosity of the macromolecular solution (η) and the radius of the macromolecule (r). $\Delta Y[Hb]_T/\Delta x$ is the critical parameter in Equation 5.6 that determines the amount of oxygen that will be offloaded over a given ΔPO_2, which is a function of the hemoglobin–oxygen equilibrium curve.

From Equation 5.6, diffusive oxygen transport also can be limited by the oxygen-carrying capacity of the cell-free hemoglobin, i.e., [*Hb*]. However, there must be a lower limit of [*Hb*] for survival. To evaluate this, a total exchange transfusion was performed in rats to compare a high-oxygen affinity, non-vasoactive cell-free oxygen carrier, maleimide-PEG-conjugated hemoglobin (MP4)

(Vandegriff *et al.*, 2003), with two non-oxygen carrying solutions: PEG-modified albumin (MPA), prepared using the same chemistry as for MP4, and 10 per cent pentastarch (PS) to match the PEG-modified proteins for oncotic activity and viscosity but without PEG (Winslow *et al.*, 2004). Continuous exchange transfusions were carried out such that the final hematocrit in all animals was between 0 and 5 per cent. All animals in the MP4 group survived (n = 5 for each group) over the 60 minutes of exchange followed by a 70-minute observation period. None of the animals that received either PS or MPA survived for more than 90 minutes. Lactic acid began to rise at ~15 per cent hematocrit in the PS and MPA groups, but at lower hematocrit (~7.5 per cent) in the MP4 group. At these points, the total [*Hb*] was ~5 g/dl in all groups. This defines a lower limit of oxygen-carrying capacity of 5 g/dl with either red blood cell hemoglobin alone or red blood cells plus an acellular, non-vasoactive, high-oxygen affinity hemoglobin. In the MP4 group, ~2.5 of the 5 g/dl were present as plasma hemoglobin, which decreased the critical hematocrit in half and provided equivalent functional oxygen delivery as red blood cells gram-for-gram of hemoglobin.

The mechanism behind hemoglobin-induced vasoconstriction has been investigated in the microcirculation during extreme hemodilution in hamsters. In those experiments, high-oxygen affinity MP4 (4.2 g/dl; *P*50 = 5 mmHg) (Vandegriff *et al.*, 2003) was compared with a low-oxygen affinity polymerized bovine veterinary product Oxyglobin™ (PolyBvHb) (13.1 g/dl *P*50 = 54 mmHg). After hemodilution, plasma hemoglobin concentrations were 1.1 and 3.7 g/dl for MP4 and PolyBvHb, respectively. The PolyBvHb solution and the red blood cells in circulation with PolyBvHb offloaded more oxygen in the systemic, arterial circulation so that by the time these oxygen carriers reached the capillary circulation, both the cellular and acellular hemoglobins were significantly more desaturated compared to MP4, or to red blood cells in circulation with MP4. MP4 preserved its oxygen saturation and that of the red blood cells in the arteries and arterioles and provided greater oxygen delivery to the capillary beds (Tsai *et al.*, 2004b). This observation describes the importance not just of oxygen-carrying capacity but of regional oxygen delivery in the microcirculation where blood flow is regulated at precapillary arterioles by innervated smooth muscle that controls the diameter of the vessels (Ping and Johnson, 1994).

FACILITATED DIFFUSION AND HYPEROXYGENATION OF ARTERIOLES

The PO_2 distribution in the microcirculation decreases from ~50 to 30 mmHg from A1 to A4 arterioles (Intaglietta *et al.*, 1996). Using these values for PO_2 and the parameters in the second term of Equation 5.6: $D_{HbO_2}(\Delta Y[Hb]_T/\Delta x)$, we can calculate a theoretical amount of facilitated oxygen transfer in arteriolar vessels in the absence of biological compensatory mechanisms. For this exercise, we will compare two well-characterized hemoglobin solutions: αα-Hb (Vandegriff *et al.*, 1997; McCarthy *et al.*, 2001) and MP4 (Vandegriff *et al.*, 2003). Since the hemoglobin solutions are acellular, we assume that the diffusion distance, Δx, is the same.

For studies of hemoglobin-induced hypertension in rats following 50 per cent exchange transfusion, αα-Hb was formulated at ~8 g/dl (Rohlfs *et al.*, 1998) and MP4 was formulated at ~4 g/dl (Vandegriff *et al.*, 2003). Upon exchange transfusion with αα-Hb, mean arterial pressure (MAP) rose and was significantly higher than baseline MAP or the MAP observed with MP4 transfusion (Figure 5.2). The slight rise in MAP for MP4 was not significantly different from baseline.

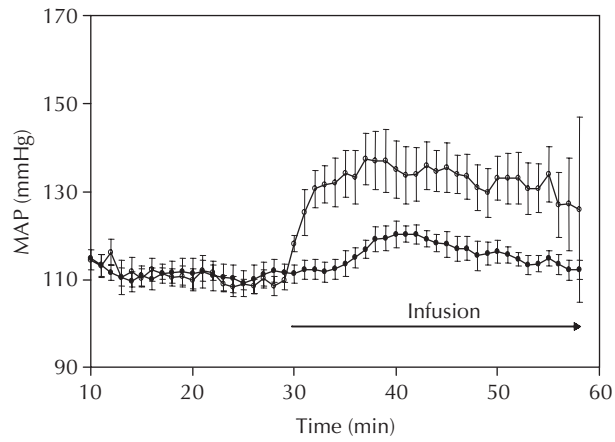

Figure 5.2 Mean arterial pressure during infusion of αα-Hb (open circles, n = 6) or MP4 (Hb-PEG-5K6) (closed circles, n = 7) in rats. The solutions were infused into the femoral vein at 0.5 ml/min. The increase in MAP upon transfusion of αα-Hb was significantly different from baseline and from MP4 MAP ($p < 0.005$). The slight rise in MAP for MP4 is not significantly different from baseline.

Table 5.1 Theoretical comparison of oxygen transfer to arterioles following a 50 per cent exchange transfusion with αα-Hb or MP4

	Arteriolar O_2 transfer (50 → 30 mmHg)	
	αα-Hb	MP4
[*Hb*] (g/dl)	4	2
Y at 50 mmHg (%)	73	95
Y at 30 mmHg (%)	43	92
Δ*Y* (%)	30	3
$\Delta[O_2]$ (ml/dl)	1.61	0.08
Diffusion constant (cm^2/s)	6×10^{-7}	0.8×10^{-7}
$\Delta[O_2]$ flow transfer ($cm^2/s \times ml/dl$)	9.7×10^{-7}	0.06×10^{-7}
ΔPO_2 (mmHg)*	537	27

* Comparable amount of O_2 that would be transported as dissolved O_2.

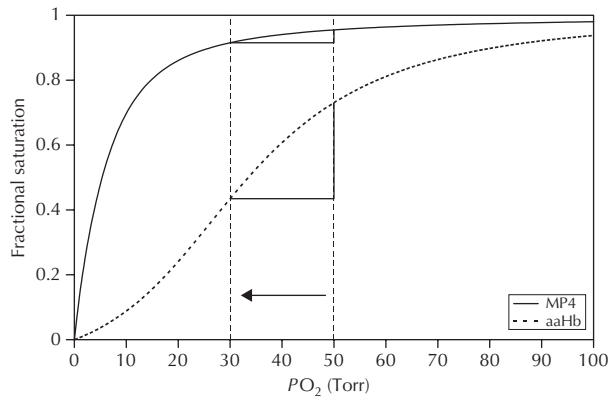

Figure 5.3 Oxygen equilibrium curves for MP4 (solid line) and αα-Hb (dotted line) at pH 7.4 and 37°C. The vertical dashed lines and arrow show the PO_2 drop in arteriolar beds (Intaglietta *et al.*, 1996). The area under the triangulated lines give the total oxygen transferred for these hemoglobins at this drop in PO_2.

If we assume a linear dilution during the 50 per cent exchange, the plasma hemoglobin concentrations would be 4 and 2 g/dl for αα-Hb and MP4, respectively, at the end of the exchange. (In fact, the MP4 plasma concentration is likely to be lower due to its hyperoncotic activity, but for the purpose of these calculations, we assume linear dilutions.) For the tetrameric hemoglobin, αα-Hb, we can use the literature value for the diffusion constant of unmodified, tetrameric hemoglobin (D_{HbO_2}) at 4 g/dl ($\sim 6 \times 10^{-7}\,cm^2/s$) (Kreuzer, 1970). D_{HbO_2} for MP4 can then be estimated from the terms η and *r* in Equation 5.3 using a 2.8-fold higher viscosity (2.5 versus 0.9 cPs) and a ~3-fold larger radius (~8 versus 3 nm) (Vandegriff *et al.*, 2003). This decreases the diffusion constant for MP4 by ~8-fold to $\sim 0.8 \times 10^{-7}\,cm^2/s$ (see Table 5.1). (The decrease in the diffusion constant for MP4 is less than that estimated for the PEG-Hb that was described in the artificial capillary experiment (i.e., 16-fold) because that PEG-Hb, obtained as a gift from Enzon, contained more PEGs conjugated per hemoglobin tetramer, making it a larger molecule and more viscous solution.)

Oxygen equilibrium curves for MP4 and αα-Hb are presented in Figure 5.3, showing fractional saturation (*Y*) as a function of PO_2 over the arteriolar drop in PO_2 from A1 to A4 arterioles. These curves provide theoretical values for the total amount of oxygen that would be offloaded ($\Delta[O_2]$) by these cell-free hemoglobins, assuming equal oxygen-carrying capacities of 1.34 ml of oxygen per gram of hemoglobin, i.e., for αα-Hb, $\Delta[O_2] = 4\,g/dl \times 0.3 \times 1.34\,ml/g = 1.61\,ml/dl$; for MP4, $\Delta[O_2] = 2\,g/dl \times 0.03 \times 1.34\,ml/g = 0.08\,ml/dl$ (Table 5.1).

Based on these calculations, the total oxygen that would be transferred by αα-Hb in A1 to A4 arteriolar beds in the absence of autoregulatory compensatory mechanisms is at least two orders of magnitude greater than that by MP4 (Table 5.1). When the $\Delta[O_2]$ values are compared to the oxygen that would be transported as dissolved oxygen (ΔPO_2) ($\alpha = 2.28$ ml/dl per atmosphere; Severinghaus *et al.*, 1972), αα-Hb presents an amount of oxygen to the arteriolar system which can be obtained physiologically only under conditions of extreme hyperoxia (i.e., 500 mmHg); in contrast, MP4 maintains a transfer of oxygen in arteriolar beds that is comparable to PO_2 levels within the normal physiological range (27 mmHg) (see Table 5.1). This evaluation is consistent with the vasoconstrictive effects of αα-Hb in contrast to the vasoinactivity of MP4, and would explain the hypertensive response that is observed with αα-Hb but not MP4 (see Figure 5.2).

Hemodynamic effects associated with the vasoconstrictive response to small, cell-free hemoglobins with low oxygen affinity are similar to those provoked as a result of hyperoxia and likely reflect a common modality of action as the result of over-delivery of oxygen to vessel walls, followed by compensatory mechanisms

to match oxygen delivery to oxygen demand (Tsai *et al.*, 2003). Vasoconstriction due to over-delivery of oxygen by hemoglobin-based oxygen carriers may occur if facilitated oxygen diffusion leads to excessive offloading of oxgen in innervated vascular beds that control vascular tone.

SUMMARY

Oxygen transport by hemoglobin, either packaged inside red blood cells or in a cell-free solution, is ruled by physical laws of molecular transport. Molecular diffusion provides a fundamental differentiation between how oxygen is transported physically by cellular versus acellular oxygen carriers or by different types of cell-free oxygen carriers. Red blood cells and intracellular hemoglobin have evolved to carry sufficient oxygen from the lungs to offload to respiring tissue. The system acts to balance oxygen delivery and oxygen demand. Vascular diameters increase to enhance oxygen delivery in hypoxic states, and decrease in hyperoxic states to avoid the inherent toxicity of oxygen. Because of oxygen's low solubility in plasma and tissues, it must diffuse some distance, which decreases down the microcirculatory network, to reach intracellular hemoglobin during oxygen uptake in the lungs or from intracellular hemoglobin to its site of consumption in peripheral tissue. Hemoglobin provides a molecular mechanism to optimize oxygen transport through the chemical reaction between oxygen and heme to form oxyhemoglobin (HbO_2).

Oxygen diffuses down its concentration gradient until its concentration is in equilibrium throughout the system, or to its site of oxygen consumption. Intra- or acellular HbO_2 will also diffuse down its concentration gradient, providing an additional mode of oxygen flux, i.e., facilitated diffusion. Hemoglobin encapsulated within the membrane space of red blood cells flowing down a vessel provides facilitated diffusion of oxygen from the center of the red blood cell to the intracellular side of the red blood cell membrane. There it releases free oxygen to diffuse through an unstirred plasma diffusion layer surrounding the red blood cell to reach equilibrium with the mixed plasma PO_2. When hemoglobin is free in solution flowing down a vessel, the diffusion barriers to HbO_2 within and surrounding red blood cells disappear and both oxygen and oxyhemoglobin diffuse freely in the intravascular space to the endothelial tissue barrier, accelerating movement of oxygen through space.

In early designs of hemoglobin-based oxygen carriers, it was considered to be effective that the carrier would match the functionality of red blood cells in equilibrium oxygen-binding properties and maintain as high an oxygen-carrying capacity as possible. The effects of facilitated diffusion and autoregulatory compensatory mechanisms were not considered. The theoretical basis of facilitated diffusion now has been demonstrated in artificial capillary systems (Page *et al.*, 1998; McCarthy *et al.*, 2001), and a correlation has been demonstrated between diffusional oxygen transport and hemodynamic responses to the cell-free hemoglobins, such that enhanced oxygen transport by facilitated diffusion is related to hemoglobin-induced hypertension (McCarthy *et al.*, 2001). The properties that affect facilitated flux in hemoglobin solutions are hemoglobin size, oxygen affinity, and solution viscosity (see Equations 5.6 and 5.3).

A new hemoglobin-based oxygen carrier in clinical development was designed with properties to match oxygen transport by the cell-free solution to that of a red blood cell suspension. The new hemoglobin product, MP4, is conjugated to polyethylene glycol to increase molecular hydrodynamic volume, have high oxygen affinity and high solution viscosity relative to tetrameric hemoglobin solutions; and it does not induce hypertension in rats (Vandegriff *et al.*, 2003), hamsters (Tsai *et al.*, 2004b), swine (Drobin *et al.*, 2004), or humans (Bjorkholm *et al.*, 2005). This new oxygen carrier has been compared at the microcirculatory level in hamsters with a polymerized oxygen carrier with lower oxygen affinity. The low-affinity oxygen carrier decreases arteriolar blood flow and FCD relative to MP4 and demonstrates that, in addition to oxygen-carrying capacity, oxygen delivery must be 'targeted' to offload sufficient oxygen in appropriate vascular beds to maintain blood flow and oxygen delivery to capillaries (Tsai *et al.*, 2004b). These results clearly show that different types of cell-free oxygen carriers act by different mechanisms of oxygen transport. These transport properties involve diffusion of the oxygen carrier, which is determined by physical properties of molecular size and oxygen binding.

EDITOR'S SUMMARY

The movement of oxygen from red blood cells to tissue cells and mitochondria follows well-defined physical laws, which are described in this chapter. What has not been well appreciated is that hemoglobin, free in solution, is also free to diffuse, and this diffusion, sometimes called 'facilitated' oxygen diffusion, has a profound effect on the regulation of vascular tone.

It is well known that the microcirculation is very sensitive to local oxygen supply; oxygen is a vasoconstrictor. Thus, the presence of oxyhemoglobin in the plasma space can oversupply tissue, which can lead to autoregulatory vasoconstriction.

While the concept of facilitated oxygen diffusion has been in the scientific literature for decades, its impact on the design of cell-free oxygen carriers has only recently been appreciated. This property can be regulated by control of key properties, including molecular size, viscosity and oxygen affinity (*P*50). Appreciation of the physical laws of oxygen supply to tissue is now leading to the design of new oxygen carriers that have reduced vasoactivity and therefore, it is hoped, toxicity.

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Chapter 6

Oxygen Transport Properties of Hemoglobin-Based Oxygen Carriers: Studies using Artificial Capillaries and Mathematical Simulation

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INTRODUCTION

Delivery of oxygen to tissue in capillaries is very different in suspensions of red blood cells mixed with extracellular hemoglobin-based oxygen carriers (HBOCs) than in unaltered blood. Put simply, HBOCs are not blood. One important way to investigate these differences is by the use of artificial capillaries. Such materials are free of biological regulatory mechanisms, and accurate determinations can be made of all parameters affecting flow and transport. A second way to investigate these differences is by mathematical simulation. Models based on fundamentals and verified by comparison with experiment can be used to interpret and extend the results of experimental measurements and to improve understanding of mechanisms of transport enhancement.

Both *in vitro* experimental methods 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 improve our understanding of 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 hemoglobin-based oxygen carriers (HBOCs), which are the focus of this work.

IN VITRO SIMULATION OF OXYGEN TRANSPORT

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-known difficulties. These difficulties are 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 (Popel *et al.*, 1989; Pittman, 1995; Hellums *et al.*, 1996; Page *et al.*, 1998a). The difficulties gave impetus to the development of the well-defined, controlled, *in vitro* systems, to be discussed later.

Mathematical simulation of oxygen transport

Mathematical simulation methods suffered for many years after Krogh's pioneering work (Krogh, 1919, 1922) from the lack of experimental data of sufficient detail and precision to critically test the proposed mathematical models. Thus,

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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 the same order as that in the surrounding tissue, and thus cannot be neglected in an accurate simulation (Hellums, 1977; Baxley and Hellums, 1983; Federspiel and Sarelius, 1984; Federspiel and Popel, 1986; Groebe and Thews, 1986; Nair *et al.*, 1989). The focus of this chapter 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 HBOCs.

Flow regimes

Significant oxygen transport occurs in microvessels of a very large range of diameters in the microvasculature. The character of the flow and, hence, the transport processes in the microcirculation are highly dependent on the diameter of the microvessel. As a result, very different approaches are required for experimental or mathematical simulation of oxygen transport in the different regimes. We recognize three distinctly different flow regimes as outlined below and illustrated in Figures 6.1 and 6.2.

Figure 6.1A is a schematic representation of erythrocytes flowing in a 25-μm diameter vessel, and gives a visual indication of the relationship between the erythrocyte dimensions and the

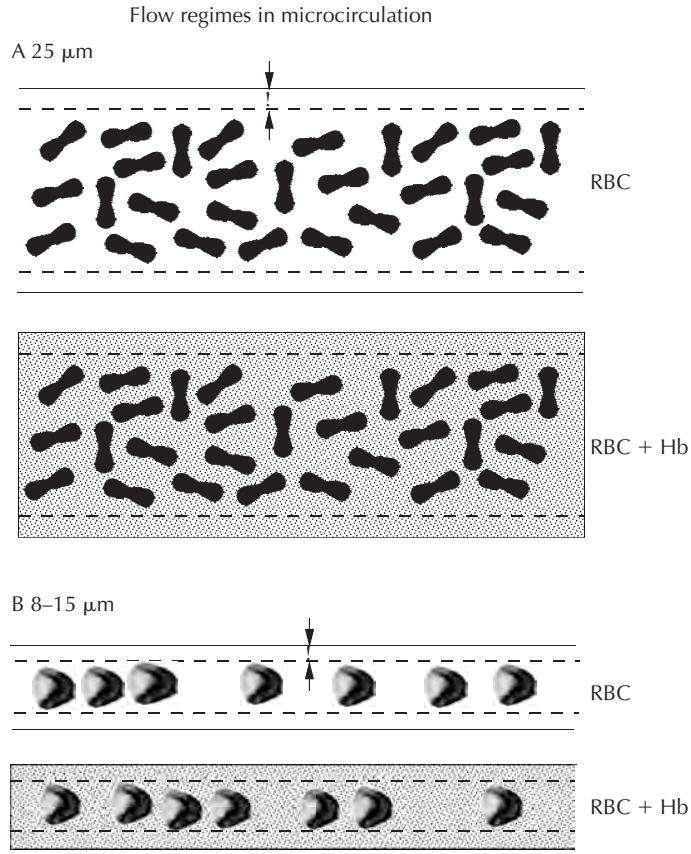

Figure 6.1 Schematic illustration of two distinct flow regimes in the microcirculation. (A) Schematic representation of the flow in 25-μm diameter vessels corresponding to arterioles. The cell-free zone is represented by δ. (B) Simplified schematic representation of flow in intermediate-sized capillaries in the range of 8–15 μm diameter. The dark dots in the background of (A) and (B) are intended to represent the presence of a hemoglobin-based oxygen carrier in the suspending medium.

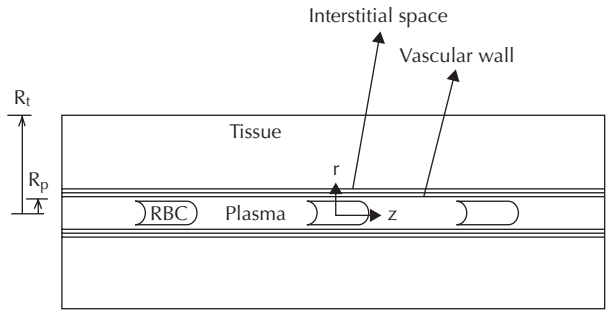

Figure 6.2 Schematic illustration of erythrocytes in 4-μm diameter capillary vessels showing computational domain with subregions RBC, plasma, vascular wall, interstitium and tissue (from Vadapalli *et al*. (2002), with permission).

conduit diameter. In vessels in this (arteriolar) size 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 (Lemon *et al*., 1987; Nair *et al*., 1989, 1990).

Figure 6.1B is a drawing (based on microscopic observations) 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 and in clusters – as a function of hematocrit. Thus, the concept of a local hematocrit that varies continuously with radial position is not applicable. Later in this chapter we will present the first *in vitro* experimental measurements of oxygen transport in this flow regime. Very little mathematical simulation work has been done on vessels is this size range. One mathematical study in this regime was performed by Wang and Popel (1983), based on the cell shapes calculated for an 8.24-μm diameter vessel by Zarda *et al*. (1977).

Figure 6.2 is a schematic representation of the third regime: that of the true capillaries with diameters in the 4–6-μm range (from Vadapalli *et al*., 2002). In these small vessels 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 (Hellums, 1980; Secomb *et al*., 1998). 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*. (1996).

CURRENT STATE OF OXYGEN CARRIER STUDIES IN ARTIFICIAL CAPILLARIES

Oxygen transport measurements have been performed on an *in vitro* artificial capillary apparatus that has been extensively described (Page *et al*., 1996, 1998a). Briefly, this apparatus is comprised of a microflow system, a dual-wavelength microspectrophotometer, a data acquisition and control system, and an environmental system. The core of one of the two microflow systems is a 120-μm thick silicone rubber film, which has been cast around a 25-μm diameter tungsten wire. A second microflow system has been formed by casting a silicone rubber film around a 10-μm diameter wire. In each case the wire is removed to produce an arteriole-sized lumen spaced equidistantly from the top and bottom of the film. Silicone rubber is an ideal material for this work because of its very high permeability to oxygen. Test samples are held in a feed reservoir at 37°C under controlled oxygen tension gas. Samples are drawn from the feed reservoir through the capillary lumen into a withdrawal reservoir by a disc encoder-controlled syringe pump. In this way stable, precisely controlled and known flow rates are produced. In a typical oxygen uptake experiment both surfaces of the film are exposed to humidified air at 37°C, and a fully deoxygenated feed sample is oxygenated as it flows through the lumen of the capillary. In a typical oxygen release experiment both surfaces of the film are exposed to humidified nitrogen at 37°C, and a fully oxygen-saturated feed sample is deoxygenated as it flows through the lumen of the capillary. An oxygen uptake experiment is represented schematically in Figure 6.3.

Samples are prepared at known pH (generally pH 7.4). Carbon dioxide transport is not measured and carbon dioxide is not used. The flow system is mounted on the stage of a Leitz UWM toolmaker microscope, which has been modified to function as a dual wavelength microspectrophotometer. A diaphragm located at the beginning of the fiberoptic light splitter is adjusted such that light from a small section of lumen is viewed by the photomultipliers at each axial position. The optical measurements are used to calculate oxygen saturations at various positions along the axis of the capillary. The environmental system

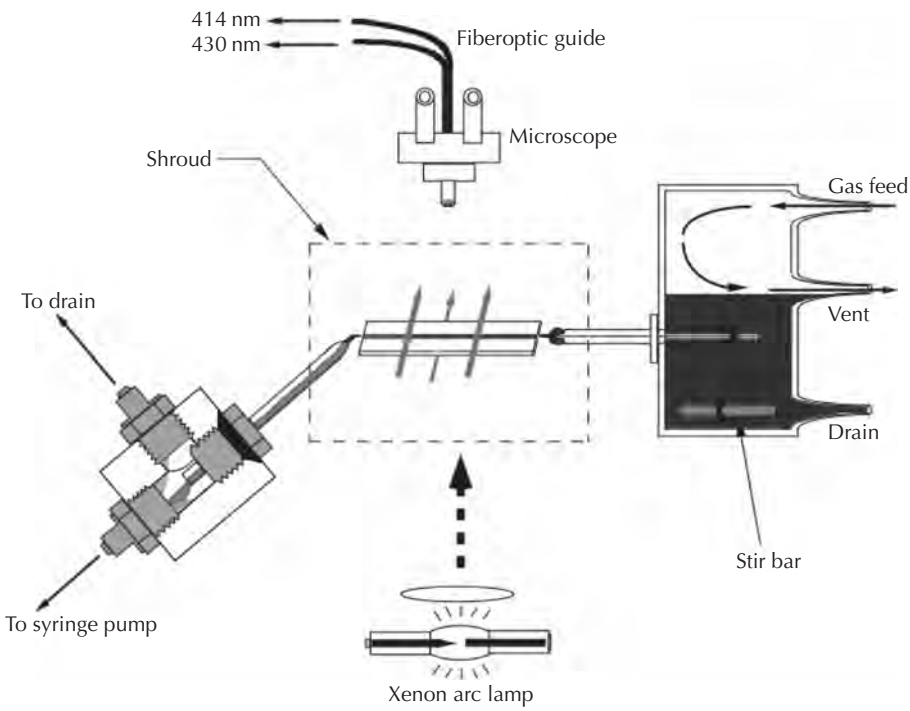

Figure 6.3 Schematic of experimental apparatus for artificial capillary – the microflow system and microspectrophotometric systems.

maintains the separate oxygen tensions in the extracapillary suffusing gas and the feed reservoir, as well as maintaining the entire system at 37°C.

A second artificial capillary system has been developed and applied by McCarthy *et al*. (2001). A 57-μm diameter silicone rubber capillary is perfused with solutions of hemoglobin and various hemoglobin-derived materials. Oxygen tension of the inlet flow and of the suffusing gas are known and controlled. Oxygen tension of the effluent material is measured, and used to determine hemoglobin oxygen saturation by use of measured dissociation curves. A finite element calculation based on a model of transport in the system is used to calculate overall mass transfer coefficients for each experiment.

CURRENT STATE OF MATHEMATICAL SIMULATION STUDIES

Capillaries of 25 μm diameter

Nair *et al*. (1989, 1990) developed a mathematical model that predicts the oxygen transport properties of red cell suspensions. Page *et al*. (1998b, 1998c) extended and improved the model to treat cases in which free hemoglobin or hemoglobin polymers (HBOCs) are in solution in the plasma phase. The improved model takes into account existence of erythrocyte and extracellular hemoglobin solution phases, radial hematocrit variation, radial velocity gradients, axial convection, radial diffusion of both oxygen and oxyhemoglobin, and shear augmentation of transport.

Results calculated by use of the model have been compared to results of experiments using the artificial capillary systems described above. In these experiments, all physical, chemical, flow and transport properties are known accurately. Furthermore, detailed oxygen fluxes and oxygen saturations are measured. Thus, comparisons with results from the experimental system can serve to test and validate mathematical models. Such validation has been difficult or impossible in many earlier studies because of the difficulty in accurate determination of all of the parameters that influence results *in vivo*.

Capillaries of 4 μm diameter

Vadapalli *et al*. (2002) have presented results from mathematical studies on oxygen transport to tissue from red cells suspended in an HBOC solution – as a model for capillary transport. The

calculations employed a red cell shape based on the calculations of Secomb *et al*. (1998). Each erythrocyte was represented as being cylindrical with a hemispherical leading end and an inverted cap trailing end, as shown in Figure 6.2. The computational domain covered five regions: the erythrocytes, the plasma (containing dissolved HBOCs), the vascular wall, the interstitium, and the surrounding tissue. The equations of motion and transport were written separately for each region, matched with appropriate interfacial conditions, and solved numerically for wide ranges of the transport and equilibrium properties.

EXAMPLES OF EXPERIMENTS AND SIMULATIONS

Capillaries of 25 μm diameter

Oxygen release and uptake results have been obtained for PolyBvHb (an investigational HBOC based on gluteraldehyde polymerized bovine hemoglobin similar to Oxyglobin, provided by Biopure, Inc., Cambridge, MA), BvHb (bovine hemoglobin solution), and human erythrocyte suspensions (red blood cells) in buffer. Additional measurements were made on mixtures of red blood cells with BvHb or PolyBvHb solutions. Samples were prepared at pH 7.4. Oxygen saturations were obtained for six axial positions along the capillary at four different flow rates ranging from 5.5 to 54 μl/h. The data were presented as hemoglobin oxygen saturation versus apparent residence time in the capillary. The apparent residence time is unique to each position and flow rate, and is calculated as the axial distance from the entrance multiplied by the cross-sectional area divided by the volumetric flow rate. By plotting the fractional saturation versus apparent residence time instead of fractional saturation versus position, the data for the four different flow rates collapse to a single curve. Thus, the use of residence time makes it possible to present a very large amount of data in a concise way. Additionally, plotting the data as fractional saturation versus apparent residence time allows comparison between different sample types over the complete saturation range and serves as an internal consistency check.

The experimental apparatus was validated by comparing oxygen uptake and release data for BvHb flowing in the capillary with predicted values obtained from a mathematical model (Lemon *et al*., 1987). The mathematical model for the homogeneous hemoglobin solution is relatively simple in comparison to models including red cells. These relatively straightforward calculations are on a firm theoretical basis, and therefore can be used to validate the experimental system.

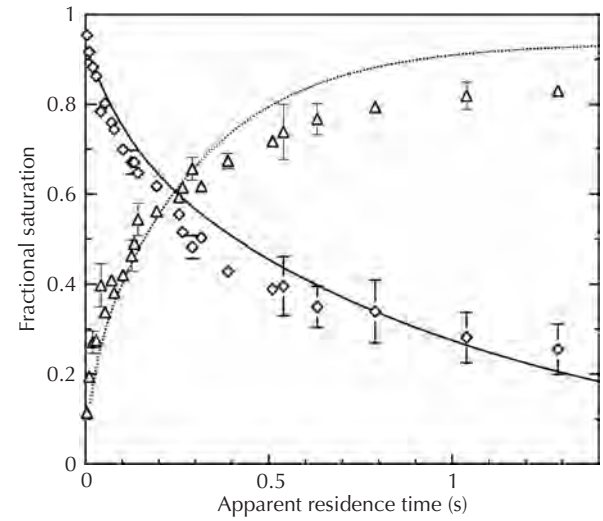

Figure 6.4 Comparison of experimental and model results for RBC/PolyBvHb 1 : 1 mixtures. Experimental data (10.2 g/dl total, pH 7.4) are plotted for uptake (triangles) and 50% oxygen gas equilibration release (diamonds). Humidified air was used to suffuse the capillary in the uptake experiments. For the release experiments, samples were equilibrated with humidified gas containing 50% oxygen. The corresponding theoretical simulations from the mathematical model described in this work are plotted as a dotted curve for uptake and as a solid curve for 50 per cent oxygen equilibration release. Shear augmentation is included in the simulations.

Extensive results of studies using a 25-μm diameter vessel have been reported by Page *et al*. (1998a, 1998b). One set of results from the work is presented in Figure 6.4. Here we see oxygen release results for a 1 : 1 (equal amount of hemoglobin intracellular and extracellular) mixture of red blood cells and PolyBvHb. Figure 6.4 also gives a comparison of red blood cells/PolyBvHb 1 : 1 experimental data with mathematical simulation. The polymerized hemoglobin exhibits a hyperbolic (low cooperativity) oxygen dissociation curve. As a result, it does not fully oxygen saturate when equilibrated with room air. To achieve full oxygen saturation, feed samples were equilibrated with a gas containing 50% oxygen. The mathematical simulation can be

seen to agree with experiments except at large residence times in uptake experiments. The simulation shows a somewhat higher uptake than the experimental data at long exposure times. This particular behavior of PolyBvHb has been discussed previously (Page *et al.*, 1998b). The polymer structure may result in a slower oxygen saturation of a percentage of the heme groups within the structure. Note that the simulations reach an asymptotic value at approximately 92 per cent saturation rather than 100 per cent. This difference is a result of the incomplete saturation of the PolyBvHb with air during typical exposure periods.

A series of calculations was carried out to predict release and uptake for formulations that were not studied experimentally. Figure 6.5 presents the results of one of these simulations. The overall hemoglobin concentration was held constant at 10 g/dl and the concentration of the extracellular hemoglobin solution was varied from 1 to 5 g/dl. Figure 6.5 shows the pronounced effects on release of altering the intracellular/extracellular ratio of hemoglobin. Addition of extracellular hemoglobin solution up to 5 g/dl is shown to enhance oxygen delivery. The curve for a hemoglobin solution of 10 g/dl overlies the curve for 5 g/dl (plus 5 g/dl red blood cell Hb) and is omitted for clarity. Thus, we see that in this case replacing half of the erythrocytes with HBOC yields essentially as much augmentation of release as replacing all of the erythrocytes.

Another series of experiments was performed by Dou *et al.* (2002). They quantified the effect on oxygen uptake and release rates of varying heme protein oxygen affinity. A series of myoglobin mutants with a wide range of oxygen affinities (500 ×range) was run through the 25-μm capillary system. The resulting data show a marked increase in the extent of oxygen release with increasing *P*50 of the test myoglobin. The myoglobin samples covered the range from full oxygen saturation to nearly full desaturation.

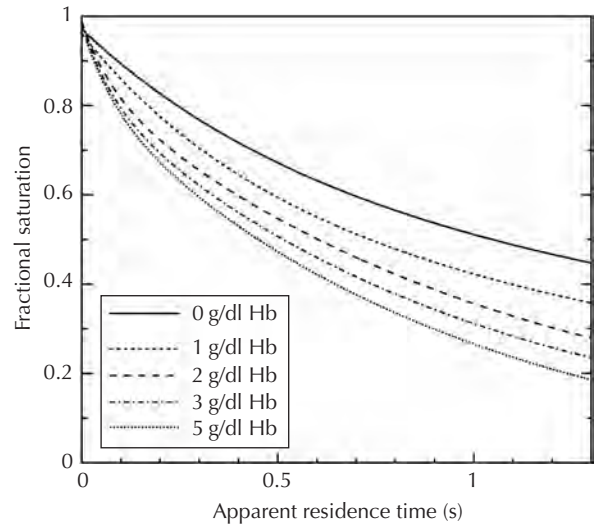

Figure 6.5 Dependence of oxygen release on extracellular hemoglobin solution concentration. Release simulations of oxygen transport by RBC/Hb mixtures with the same overall hemoglobin concentration are shown as curves. Results are for an RBC *P*50 of 27 mmHg and an Hb *P*50 of 25 mmHg. The plot legend notation of 1 g/dl extracellular hemoglobin solution, etc., refers to the extracellular hemoglobin concentration. The 0 g/dl extracellular hemoglobin solution curve refers to a red blood cell suspension. The pure (10.2 g/dl) Hb solution simulation is not shown because the results are indistinguishable from the 5 g/dl Hb curve.

Capillaries of 10 μm diameter

Data for oxygen uptake and release by bovine hemoglobin solutions (BvHb) solutions flowing in a 10-μm capillary are shown in Figure 6.6 in comparison with results from a mathematical simulation. As in the case of the larger vessel, the calculated values for hemoglobin solutions serve to validate the experimental system.

Similar results for red blood cell (RBC) suspensions are shown in Figure 6.7. As expected, oxygen uptake occurs more quickly than oxygen release due to the larger oxygen tension gradient in the uptake experiments.

The results of dose–response experiments for oxygen release by RBC/BvHb mixtures are summarized in Figure 6.8. Four data series of the same overall hemoglobin concentration (10 g/dl) with the varying hemoglobin distribution are plotted. The hemoglobin distribution varied from 100 per cent in solution (BvHb) to 100 per cent within the red blood cells (RBC). There were two intermediate solutions with RBC/BvHb mixtures. The oxygen equilibrium curves for the erythrocyte and extracellular hemoglobin are approximately the same (same *P*50 and cooperativity). The extracellular hemoglobin oxygen affinity was purposely selected to be equal to the erythrocyte affinity so that effect of encapsulated versus free hemoglobin on oxygen transport

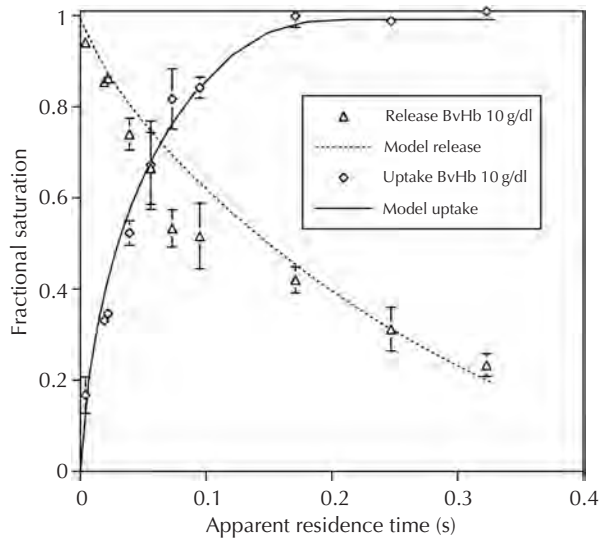

Figure 6.6 Hemoglobin solution oxygen uptake and release experiments for a 10-μm diameter capillary. Experimental determinations of hemoglobin oxygen saturation are presented as a function of residence time in the capillary. The data points represent measurements at five axial positions at two different flow rates. The error bars denote the standard deviation for replicate experiments. The curves are from predictive theoretical calculations. The good agreement between the experimental and theoretical values serves as a validation of the experimental apparatus.

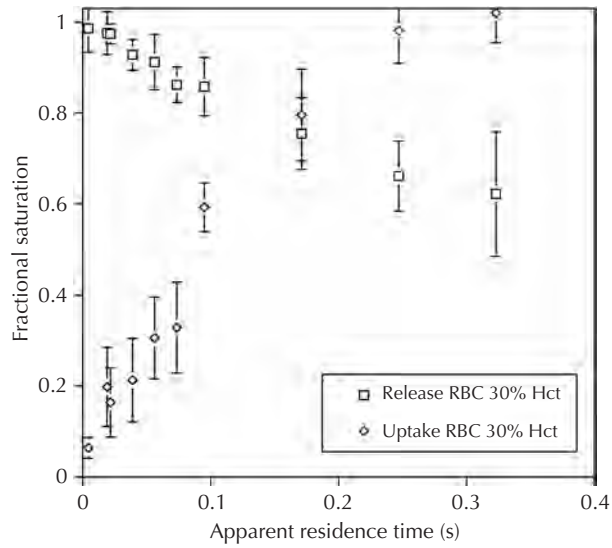

Figure 6.7 Erythrocyte suspension oxygen uptake and release experiments for a 10-μm diameter capillary. Fractional saturation is plotted as a function of apparent residence time in the capillary. Both uptake (diamonds) and release (squares) are shown. The error bars denote standard deviation. Error bars are larger for erythrocyte suspensions than for hemoglobin solutions due to the sieving effect where light may pass through variable concentrations of hemoglobin, including passing through plasma gaps. The sieving effect tends to reduce the signal-to-noise ratio.

could be studied. The least efficient oxygen transport was by the pure red blood cell suspension, and the most efficient was by the pure BvHb solution and the RBC/BvHb 1:1 mixtures. For the purposes of clarity, the data for pure BvHb and pure RBC are shown as curves without the corresponding data points. The omitted data points may be seen in Figures 6.6 and 6.7.

The intermediate data series in Figure 6.8 are for an RBC/BvHb 9:1 mixture. In this case, with only 10 per cent of the hemoglobin in the extracellular space, oxygen release was significantly enhanced over red blood cell suspensions. The RBC/BvHb 1:1 mixture was even more efficient. The data for this equal part mixture are almost indistinguishable from the 100 per cent BvHb data.

Capillaries of 57 μm diameter

McCarthy *et al*. (2001) used their artificial capillary system to evaluate oxygen release performance of erythrocytes, unmodified hemoglobin (Hb), crosslinked hemoglobin (αα-Hb), and bovine hemoglobin conjugated to polyethylene glycol (PEG-PvHb). The Hb and αα-Hb released oxygen at substantially higher rates than the erythrocytes and PEG-BvHb. The PEG-BvHb delivered oxygen at approximately the same rates as the erythrocyte suspension.

Capillaries of 4 μm diameter

Vadapalli *et al*. (2002) presented very extensive results of calculations of oxygen transport to tissue from a 4-μm diameter capillary for erythrocyte–HBOC mixtures. They explored the effects of several parameters, including inlet oxygen tension, HBOC oxygen affinity, HBOC oxygen cooperativity, HBOC concentration, erythrocyte hematocrit, and hemoglobin kinetic dissociation rates. They also investigated aspects of their model, including the importance of plasma convection, and HBOC-facilitated diffusion. Results are obtained

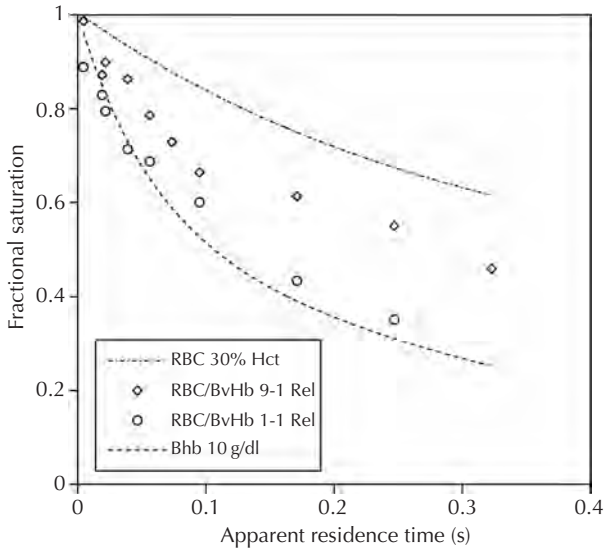

Figure 6.8 Dose response release plot for RBC/BvHb mixtures. Release summary results for red blood cell suspensions, BvHb solutions, and RBC/BvHb mixtures show the effect on oxygen release of varying the extracellular to intracellular hemoglobin ratio. 1 : 1 mixtures have 50 per cent intracellular and 50 per cent extracellular hemoglobin; 9 : 1 mixtures are 90 per cent intracellular hemoglobin and 10 per cent extracellular hemoglobin. The total hemoglobin content is 10 g/dl in all cases. Intracellular hemoglobin has a *P*50 of 27 mmHg and the extracellular BvHb has a *P*50 of 25 mmHg. Data series for red blood cell suspensions (dot–dash) and BvHb solutions (dash) are presented as best-fit curves for clarity.

in detail including hemoglobin oxygen saturations and oxygen tension distributions. Most of the results are expressed in the form of cell and capillary mass transfer coefficients. These transfer coefficients are directly related to average oxygen delivery rates for a given oxygen tension. Thus the coefficients can be used to predict oxygen delivery and axial distributions of oxygen saturation and oxygen tension under various circumstances. Typical results from this work show dramatic increases in mass transfer coefficient induced by increasing HBOC concentrations.

IMPLICATIONS FOR *IN VIVO* OXYGEN TRANSPORT

The use of both *in vitro* experiments and computational simulation for the study of oxygen transport to tissue can improve our understanding of basic physiological and pathophysiological processes. Furthermore, such studies are of particular interest in cases involving HBOCs – where the oxygen transport properties differ from unaltered blood.

As shown in the section above entitled Flow regimes, the methods of study needed are very different in different parts of the microcirculation. In the larger-diameter microcirculation, vessels of 20 μm diameter and larger, it is possible to perform accurate simulations of oxygen transport events by use of mathematical models which invoke the simplifying idealization of a hematocrit that varies continuously with radial position. Furthermore, in vessels of this size range it is possible to create artificial capillaries of silicone rubber. Silicone rubber has two key properties: extremely high oxygen permeability, and low optical density. Thus, accurate measurements of oxygen delivery processes can be made by microspectrophotometric methods in cases in which all flow and transport parameters are controlled and known accurately.

For smaller diameters, in the range of 10 μm, the character of the flow is completely different. The individual cells are significantly deformed, and pass along in single file or in clusters as a function of hematocrit. For experimental simulation it is possible to use silicone rubber capillaries in much the same way as in larger vessels. However, the computational simulation is more challenging. Few attempts have been made in the mathematical approach, and there has been no validation of computed results by comparison with detailed, accurate experimental measurements.

For still smaller diameters, in the range of 4 μm, the computational situation is simpler because the cells are highly deformed into an almost cylindrical shape. The flow is regular, laminar, and at a low Reynolds number, so accurate computational simulation is possible. However, the *in vitro* experimental possibilities are severely limited. Such measurements would require a different oxygen saturation detection system than the one described here. Furthermore, the mechanical properties of silicone rubber, coupled with the inherent difficulty of maintaining steady, measured flows and oxygen fluxes in such small silicone rubber vessels, have prevented accurate experimental measurements *in vitro*. Though experimentally challenging, this flow regime is obviously important. In red blood

cell suspensions most oxygen transport in these small vessels occurs through the vessel wall in close proximity to the red cell. Thus, the 'effective' surface area for oxygen transport can be less than half of the total capillary surface area, and it decreases with decreasing hematocrit. The addition of HBOC 'recovers' the unused surface area.

Oxygen uptake and release rates for both BvHb solutions and red blood cell suspensions were much higher in 10-μm capillaries than in 25-μm vessels. This difference might be expected due to the 2.5 times higher surface-to-volume ratio in the smaller vessels. A comparison of oxygen uptake data for 10-μm and 25-μm vessels is presented in Figure 6.9.

In the 10-μm capillary, full saturation for BvHb was approached at a residence time of approximately 0.3 seconds while full saturation required 0.7 or more seconds in the 25-μm capillary. In addition to the absolute increase in oxygen uptake rates, there was an increase in the difference between oxygen uptake rates for red blood cell suspensions and BvHb solutions in the smaller-bore vessels. Note the lengths of the arrows designating the difference between the fractional saturation of the BvHb solution and the red blood cell suspension for the different sized vessels. Both arrows are positioned where the fractional oxygen saturation of the BvHb solution is 80 per cent. In the 10-μm capillary at this residence time approximately 80 per cent more oxygen is taken up by BvHb solutions than by red blood cell suspension. In the 25-μm capillaries, approximately 65 per cent more oxygen is taken up by BvHb than by the red blood cell suspension.

A comparison of oxygen release data for 10-μm and 25-μm vessels is presented in Figure 6.10. In the 10-μm capillary 60 per cent fractional oxygen saturation of red blood cell suspension was approached at a residence time of approximately 0.3 seconds, while the same change required about 0.8 seconds in the 25 μm capillary. As in the case of oxygen uptake, there was an increase in the relative difference between oxygen uptake

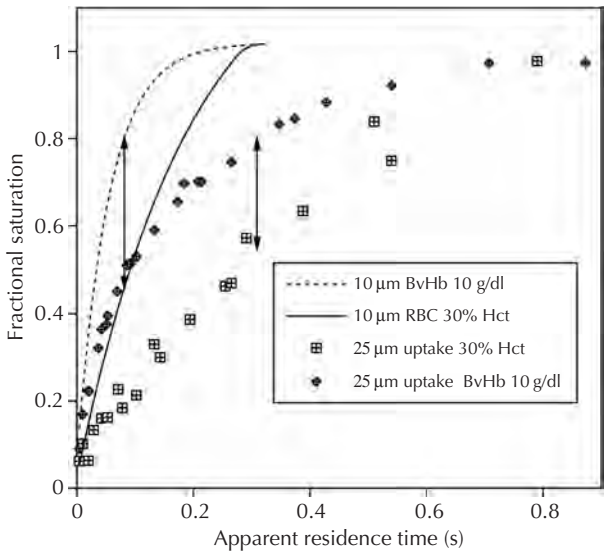

Figure 6.9 Effect of vessel diameter on oxygen uptake by BvHb and RBC suspension. Oxygen uptake for BvHb (10 g/dl) and RBC suspension (30 per cent Hct) are plotted versus apparent residence time for 10-μm and 25-μm capillaries. Data for the 10-μm capillary are shown as fitted curves for clarity. The arrows show the difference between BvHb and RBC for each diameter vessel. The arrows are drawn where the fractional saturation of the BvHb is 0.80. The difference is larger for the 10-μm capillary than the 25-μm capillary.

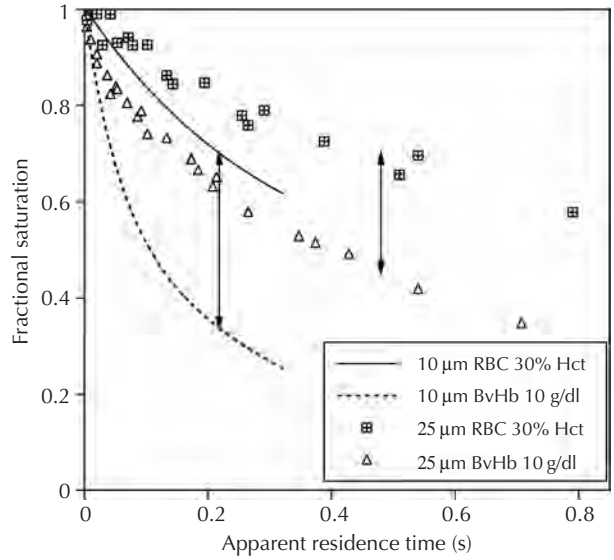

Figure 6.10 Effect of vessel diameter on oxygen release by BvHb and RBC suspension. Oxygen release for BvHb (10 g/dl) and RBC (30 per cent Hct) are plotted versus apparent residence time for 10- and 25-μm capillaries. Data for 10-μm capillary are shown as fitted curves for clarity. The arrows show the difference between BvHb and RBC for each diameter vessel. The arrows are drawn where the fractional saturation of the BvHb is 0.70. The difference is substantially larger for the 10-μm capillary than for the 25-μm capillary.

rates for red blood cell suspensions and BvHb solutions in the smaller-bore vessels. Note the lengths of the arrows designating the difference between the fractional saturation of the BvHb data and the red blood cell data for the different sized vessels. Both arrows are positioned where the fractional oxygen saturation of the BvHb solution is 70 per cent. In the 10-μm capillary at this residence time approximately 100 per cent more oxygen is released by BvHb solutions than for the red blood cell suspensions. In the 25-μm capillaries approximately 70 per cent more oxygen is released by BvHb than by the red blood cell suspension despite equivalent hemoglobin oxygen affinity.

For vessels in the true capillary range of diameters, Vadapalli *et al*. (2002) presented extensive results of calculations of oxygen release in the presence of HBOCs. Typical results for several cases of addition of HBOC to red blood cell suspensions are shown in Figure 6.11. The ordinate is the mass transfer coefficient, k_o. This coefficient is proportional to the oxygen release rate for a given oxygen tension difference. It can be seen that HBOC addition greatly increases the mass transfer coefficient.

It has been shown that HBOCs can substantially increase oxygen delivery when compared to normal blood in all regimes of the microcirculation. However, there are some modified HBOCs that do not deliver oxygen at higher rates than red blood cells. In the experiments of McCarthy *et al*. (2001), PEG-BvHb delivered oxygen at a rate very similar to that of red blood cell suspensions of the same overall hemoglobin content. The PEG-BvHb used in the experiments had an oxygen *P*50 of 10.2 mmHg, whereas the red blood cell suspension had a *P*50 of 28.6 mmHg. Our calculations of oxygen release for the conditions of their experiments using the model discussed above (Page *et al*., 1998b) are consistent with their findings for PEG-BvHb, red blood cells and αα-Hb. Our calculations for the case of HbA_0 indicate lower rates of release than the experimental results. The agreement in calculated release for red blood cell suspensions and PEG-BvHb indicates that the relatively low rate of oxygen release for PEG-BvHb is due to its high oxygen affinity. Using a high oxygen affinity HBOC offsets the tendency of HBOCs to deliver oxygen rapidly. A lower rate of delivery of oxygen by a HBOC is regarded as favorable by McCarthy *et al*. (2001), and by others (e.g. Intaglietta *et al*., 1996). None of the *in vitro* experimental or computational methods discussed here account for changes due to *in vivo* regulatory responses.

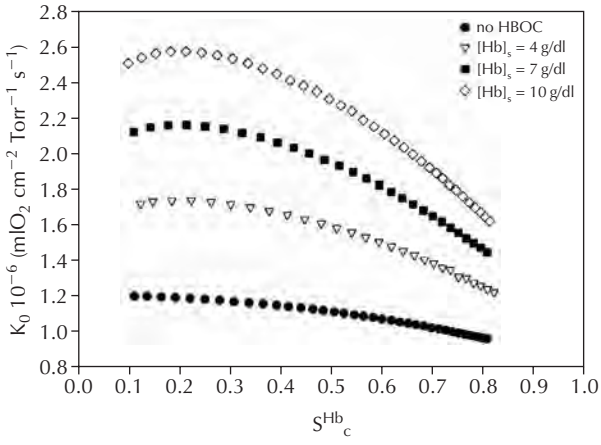

Figure 6.11 Effects of HBOCs on oxygen transport in 4-μm diameter capillaries by comparison of k_0 (mass transfer coefficient) for different values of HBOC concentrations. The *P*50 of hemoglobin was 29.3 mmHg both in the cells and in the HBOC (adapted from Vadapalli *et al*. (2002), with permission).

SUMMARY

Determination of the oxygen transport properties of HBOCs requires different approaches for the three different regimes of flow in the microcirculation corresponding to three different ranges of microvessel diameter. An outline of experimental *in vitro* and computational methods for determining these oxygen transport properties is given, along with typical results from the various approaches. These approaches make it possible to estimate the effects of various parameters associated with oxygen transport, including vessel diameter, flow rate, hematocrit; and HBOC concentration, oxygen affinity, and cooperativity.

The main features of the models and examples

1. Results are given from oxygen uptake and release experiments on suspensions of red blood cells in buffer containing various amounts of extracellular HBOCs flowing in a 25-μm diameter silicone rubber microvessel.

In this case there is a well-developed mathematical simulation method and results of the simulations are given along with the experimental results (Page *et al.*, 1998a, 1998b) Results are also discussed from experiments performed using a 57-μm diameter silicone rubber vessel (McCarthy *et al.*, 2001).

2. Similar results are given from oxygen uptake and release experiments in a 10-μm diameter capillary system. Mathematical simulation results are not available for flows in this regime.
3. Results are presented for the third regime: that of small capillaries – in the order of 4 μm diameter. In this case *in vitro* experimental results are not available, but there is a well-developed mathematical simulation method.

The experimental and mathematical models show that both hemoglobin solutions and HBOC solutions were substantially more efficient than erythrocyte suspensions in uptake and release of oxygen for similar *P*50 over all three flow regimes. Increased extracellular hemoglobin concentration increased oxygen transport rates for both uptake and release, even when total hemoglobin concentration was held constant. When only 10 per cent of the total hemoglobin was extracellular, approximately half of the increased efficiency of pure hemoglobin solutions was reached. When 50 per cent of the total hemoglobin was extracellular, the increased efficiency was virtually equal to that of pure hemoglobin solution.

As McCarthy *et al.* (2001) showed, manipulation of the hemoglobin molecule to yield a high oxygen affinity HBOC can yield oxygen transport rates similar to that of unaltered blood in arteriolar-sized vessels. Some evidence indicates that the lower oxygen delivery in the arterioles is desired.

There are two competing paradigms of oxygen transport by HBOCs that can be summarized as equilibrium versus dynamic. Design of first generation HBOCs was guided by the effort to reproduce the oxygen equilibrium curve for intracellular erythrocyte hemoglobin so that at oxygen tensions present in the lung and tissue oxygen would be delivered in similar quantities on a per-heme basis. Generally this can be called the 'blood substitute' or equilibrium model. The experimental and mathematical modeling efforts reviewed here show that the transport characteristics of HBOC solutions differ significantly from red blood cell suspensions. Thus, the dynamic or 'oxygen therapeutic' model can be used to target HBOC material properties to yield oxygen delivery similar to that of red blood cell suspensions in the various microvessels, or to yield enhanced oxygen delivery to tissue.

EDITOR'S SUMMARY

Early attempts to design artificial cell-free oxygen carriers attempted to duplicate key properties of red blood cells, including their oxygen affinity (*P*50) based on the assumption, taken from red cell physiology, that lower affinity might increase tissue oxygenation. Experiments with artificial capillaries, as discussed in this chapter, lead to the opposite conclusion.

Artificial capillaries can be constructed using materials that simulate the oxygen transport conditions in microvessels of various sizes, independent of the mechanisms that control vascular diameter (vasoconstriction). This is critically important in understanding the properties of cell-free oxygen carriers, red blood cells, and mixtures of the two. The experimental methods, models and mathematical simulation confirm that cell-free hemoglobin is much more efficient in delivering its oxygen to vessel walls than are red blood cells. Furthermore, oxygen transfer from red blood cells to vessel walls is more efficient as well when they are combined with a cell-free oxygen carrier.

Taken together with the current understanding of the *in vivo* regulation of vascular tone, it is clear that the optimal design of a cell-free oxygen carrier must take into account that if oxygen is too readily available, the paradoxical response of vasoconstriction would be expected. Thus, the design of a cell-free oxygen carrier that does not engage regulatory vasoconstriction may include such unexpected properties as increased, rather than decreased, oxygen affinity.

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Chapter 7

Mechanisms of Oxygen Transport in the Microcirculation: Effects of Cell-Free Oxygen Carriers

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INTRODUCTION

Blood exerts its principal functions in the microcirculation, the locale of exchange of the materials that it transports, and therefore introduction of a blood surrogate must insure efficacy at this level. Engineering uses the concept of 'impedance matching' to describe the process that insures that efficiency in the transmission of energy and signals between systems is maximal. We propose that the same concept is applicable when considering how to devise a fluid to replace blood, particularly if this fluid has different hydraulic and transport properties. This 'matching' is critical and a sort of moving target in the microcirculation, because the system of conduits rapidly reacts and adapts to the changed blood properties to maintain the supply of oxygen at a constant level.

Since its discovery, the microcirculation has been viewed as the system that allows atmospheric oxygen to be brought within a short diffusion distance of the cellular components of an organism where direct diffusion is not sufficient. This view has expanded considerably, since it is presently apparent that many disease processes originate in the microvascular endothelium. Furthermore, while it was previously thought that capillaries were the principal suppliers of oxygen to the tissue, information on the distribution of oxygen tension in the microcirculation obtained using new techniques has shown that arterial vessels are also major contributors to this process because the vessel wall is not a barrier to the exit of oxygen, and arterial blood provides the largest oxygen concentration gradients – the driving force behind oxygen delivery from blood into the tissue.

The primary function of both the micro- and the macro-circulation is undoubtedly the supply of oxygen to the tissues, a process whose energy requirement is assumed to be circumscribed mainly by the energy expenditure of the heart and lungs. The energy expenditure of a blood vessel, as tissue that requires oxygen for its metabolism and expends energy in regulating blood flow, is assumed to be similar to that of most tissues. In this conceptualization, the maintenance of vascular tone is aided by a 'ratchet mechanism' in smooth muscle by which steady constriction is sustained without the expenditure of energy.

These widely held generalizations are challenged by data derived from *in vivo* experiments in the microcirculation on the gradients of oxygen tension in the perivascular tissue and their application to the measurement of oxygen delivery and consumption at the microscopic level. These

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findings have implications in deciding how to design an oxygen carrier that substitutes red blood cells and are discussed in this chapter in terms of oxygen transport, while the effects resulting from altered blood viscosity are discussed in Chapter 8.

THE DISTRIBUTION OF OXYGEN IN THE CIRCULATION

Data on longitudinal oxygen distribution along the circulation and the microcirculation show that arteriolar blood vessels of most tissues release a significant amount of oxygen, with the result that in general the oxygen supplied by capillaries is secondary to that provided by the arterioles. This arteriolar release of oxygen determines a longitudinal oxygen gradient in the circulation, as shown by Tsai *et al*. (2003). It has been well established, since the studies of Duling and Berne (1970), that the circulation strives to maintain this pattern of oxygen distribution by adjusting flow via a change in caliber of the resistance arterioles. Vasodilatation increases blood availability while maintaining a relatively constant rate of oxygen exit, and *vice versa*. Although the specific nature of the microvascular oxygen sensor is not yet fully established, adrenergic enervation in the arteriolar wall is an important component of the mechanism that controls arteriolar tone, modulating flow in such a fashion that the pattern of oxygen distribution remains approximately normal. It is notable that the steepest portion of the oxygen dissociation curve corresponds to the location of the highest adrenergic nerve endings in the microcirculation (Saltzman *et al*., 1992).

Changes in the composition of blood, its viscosity, and the oxygen dissociation curve for hemoglobin generate signals that are important in designing and using blood substitutes. A decrease in the oxygen-carrying capacity due to hemodilution induces vasodilatation, and the restoration of the lost red blood cells with an oxygen carrier can produce an additional set of signals depending on whether the carrier has a high or low affinity for oxygen. A high oxygen affinity (low *P*50) results in oxygen being preferentially unloaded in the capillaries, and *vice versa*. An additional factor that regulates the delivery of oxygen to the tissue is the consumption of oxygen by the vessel wall. Such consumption is usually assumed to be similar to that of the parenchyma, but several studies show that it may be extraordinarily high, and that it increases with vasoconstriction (Ye *et al*., 1990; Friesenecker *et al*., 2004).

Figure 7.1 shows the normal pattern of oxygen distribution in the microcirculation of the tissue in the chamber window preparation of the hamster. It also shows, for comparison, the pattern of oxygen distribution after treatment with arginine vasopressin, a vasoconstrictor, and the same distribution after treatment with a vasodilator that reduces the vessel wall oxygen consumption to the theoretical lower limit, being similar to that of the surrounding tissue. The significance of these configurations is that the *tissue is not supplied with oxygen by blood but by the blood vessels*, and therefore their oxygen consumption is a factor.

OXYGEN DELIVERY AND CONSUMPTION

The amount of oxygen that arrives in the microcirculation and its release from it can presently be calculated at the microscopic level. The hamster window chamber provides a suitable model for carrying out this analysis because the tissue is isolated from the environment and is not subjected to problems due to the suffusion necessary in exposed preparations (Endrich *et al*., 1980). These calculations rely on a series of measurements of the transport properties of blood and microvessels, including the flow velocity, vessel diameter, local hematocrit level and hemoglobin plasma concentration, and require information describing the oxygen dissociation curve for hemoglobin.

Two oxygen balances can be made, one describing pre-microvascular oxygen delivery and the other the actual oxygen released from blood into the tissue. The oxygen-carrying capacity of blood is determined from the level of hematocrit, and a systemic measurement suffices for the calculation pre-microvascular oxygen delivery. Hematocrit in the microcirculation decreases as a function of blood vessel size, and the correction developed by Lipowsky and Firrell (1986) must be used for comparing pre- and microvascular oxygen delivery.

Equations 7.1 and 7.2 give the $O_{2\ \text{delivery}}$ (defined as the amount of oxygen per unit time delivered by the arterioles to the microcirculation normalized relative to control) and $O_{2\ \text{extraction}}$ (defined as the amount of oxygen released by blood in the

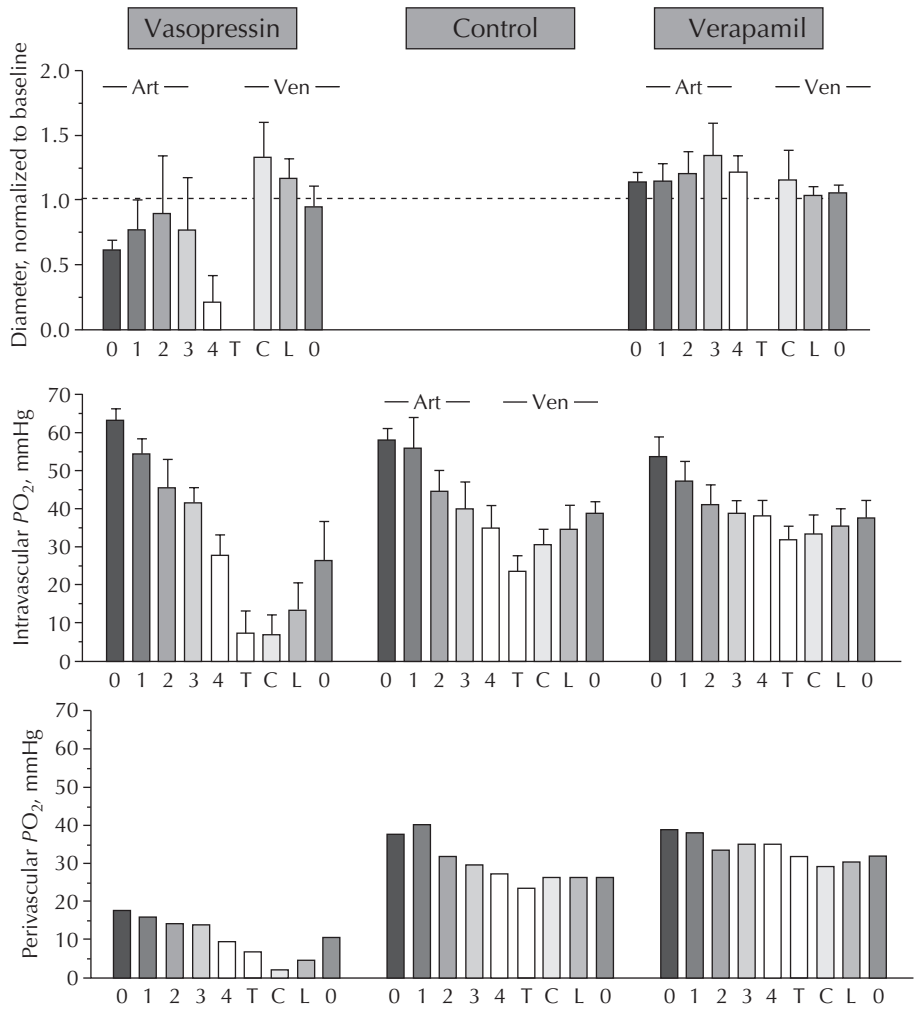

Figure 7.1 Distribution of oxygen tension in the microcirculation as function of vessel order, starting with large A0 arterioles (diameter ~ 100 μm) and ending with large V0 venules (diameter 200 μm) in the hamster window model. Data presented are for normal control conditions, and after treatment with a vasodilator (Verapamil) and with a vasoconstrictor (Vasopressin). The vessel wall gradient is the fall of PO_2 across the vessel wall, which is a function of oxygen consumption by the tissue. The lower panel shows the oxygen tension outside each blood vessel order, namely the intravascular PO_2 minus the vessel wall gradient, or the oxygen tension 'seen' by the tissue, illustrating the concept that the tissue derives its oxygen supply from the blood vessels, and indirectly from blood.

microcirculation per unit time normalized relative to control):

$$O_{2\ \text{delivery}} = [(RBC_{Hb} \times \gamma \times S_A\,\%) + (Plasma_{Hb} \times \gamma \times \hat{S}_A\,\%) + (1 - Hct) \times \alpha \times PO_{2\,A}] \times Q \quad (7.1)$$

$$O_{2\ \text{extraction}} = [(RBC_{Hb} \times \gamma \times S_{A-V}\%) + (Plasma_{Hb} \times \gamma \times \hat{S}_{A-V}\,\%) + (1 - Hct) \times \alpha \times PO_{2_{A-V}}] \times Q \quad (7.2)$$

where RBC_{Hb} is the hemoglobin in red blood cells expressed in g/dl of blood; $Plasma_{Hb}$ is the cell-free hemoglobin in g/dl of blood; γ is the oxygen-carrying capacity of hemoglobin at 100 per cent saturation or 1.34 ml O_2/g Hb; S_A% is the arteriolar oxygen saturation of red blood cells; $\hat{S}_{A-V}$% is the arteriolar oxygen saturation of the oxygen carrier where the subscript A − V indicates the difference between arterioles and venules; $(1 - Hct)$ is the fractional plasma volume and converts the equation from per dl of plasma

to per dl of blood; α is the solubility of oxygen in plasma equal to 2.14×10^{-3} ml O_2/dl plasma mmHg; $PO_{2\,A}$ is the arteriolar partial pressure of oxygen, $PO_{2\,A-V}$ is the arteriolar/venular difference in PO_2; and Q is the microvascular flow for each microvessel as a percentage of baseline. The hemoglobin saturation is determined from the PO_2 and using the oxygen dissociation curve for hamster blood, which can be calculated according to the algorithm of Winslow *et al*. (1983), or measured with the Hemox Analyzer (TCS Corp., PA), provided that the curve is corrected for the Hb saturation at 150 mmHg. This equation can also account for the addition of oxygen carriers requiring information on their oxygen dissociation curve vs PO_2 and their concentration in blood.

Analysis of the oxygen transport efficacy of blood substitutes at the level of the microcirculation with the hamster window chamber model may be considered to yield a somewhat restricted perspective, considering that hamsters belong to a fossorial species with unusual normal blood gases (namely arterial $PO_2 \sim 60$ mmHg), and that the window tissue is part of a skin fold. Logistically a rat window chamber model would be more suitable, and mice may be an alternative. However, rat window chamber models, originally developed by Hutchins *et al*. (1988), have so far not been effective in large-scale studies, being prone to infection. The aggressive behavior of this species also leads to determined attempts to removing their window construct. Mice pose a surgical challenge due to their small size. It is a general misconception that the tissue in the hamster window chamber is skin; it comprises subcutaneous connective tissue, muscle and adipose tissue, and therefore is representative of the major tissue masses in the body. As an example, intravital microscopy of the small intestinal microcirculation during hemorrhagic hypotension shows vasoconstriction of inflow (first-order) and premucosal (third-order) arterioles, but compensatory dilation of seromuscular arterioles (fourth order) (Gosche and Garrison, 1991). This is the same pattern of responses found in the chamber window preparation in response to hemorrhage (Colantuoni *et al*., 1985).

ANEMIA AND HYPEROXIA

In most instances blood substitutes would be used to restore oxygen supply to tissue that has presumably been reduced to hypoxia due to limitation of the oxygen-carrying capacity owing to decreased hemoglobin content. The microvascular responses to this condition have been repeatedly explored in studies of extreme hemodilution with non-oxygen-carrying plasma expanders. Decreased oxygen availability is a condition that leads to vasodilatation, which is a well-known response. As anemia increases owing to further dilution of the red blood cell mass, the usual finding is that blood pressure is reduced, and a concomitant reflex vasoconstriction aimed at maintaining blood pressure decreases functional capillary density (FCD) and microvascular function. This effect was thought to be due to reduced cardiac function owing to the lowered oxygen supply; however, recent studies have shown that this phenomenon is due to the significantly reduced blood viscosity, which depressurizes the microvasculature, and which is reversed by increasing plasma viscosity without increasing oxygen-carrying capacity (Tsai *et al*., 1998; Cabrales *et al*., 2004a).

Hyperoxia is equally important in the design of blood substitutes, since this condition causes vasoconstriction (Figure 7.2) and can arise locally as a consequence of the altered changes of blood oxygen transport properties, and in the absence of excess oxygen inspiration by the lungs. In this context hyperoxia should be viewed in terms of the oxygen sensed by the arteriolar wall – a factor that is affected by the shape of the oxygen dissociation curve of hemoglobin and the presence of molecular hemoglobin in plasma. Low affinity hemoglobin will unload significant amounts of oxygen in the pre-capillary circulation, while the presence of molecular hemoglobin in plasma forms an additional flux of oxygen from the blood column to the vessel wall by the process of facilitated diffusion (McCarthy *et al*., 2001).

The physiological responses to anemia, and the potential for causing vessel wall hyperoxia, play a role in defining the properties of a blood substitute. Furthermore, these effects are seldom isolated, usually forming part of a multifactorial process – as shown by the example of the relationship between anemia, cardiac function and viscosity.

THE HEMOGLOBIN – OXYGEN EQUILIBRIUM CURVE

Autoregulation of the oxygen supply insures that the organism adapts to moderate changes

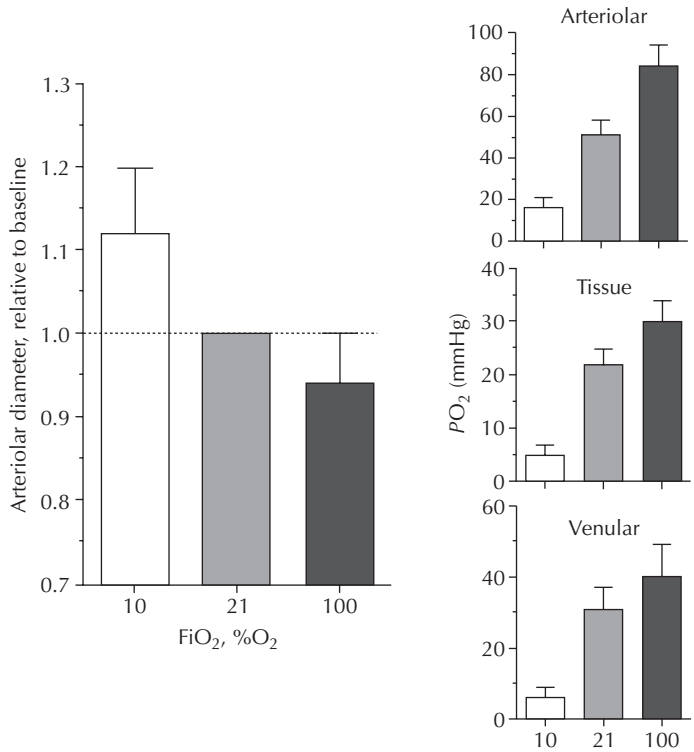

Figure 7.2 Arteriolar diameter in conditions of hypoxia, normoxia and hyperoxia caused by changing the composition of inspired gases or FiO_2. Changes from normal air inspiration determine significant changes in tissue perfusion as the microcirculation strives to regulate oxygen delivery.

in blood properties, and therefore the efficacy of a blood substitute cannot be determined unequivocally unless it is tested in conditions that are outside this compensatory process. Extreme hemodilution is an experimental model used to accomplish this. It consists of reducing systemic hematocrit to the point where oxygen delivery becomes dependent on the intrinsic oxygen-carrying capacity of the circulation. This point is reached by means of isovolemic, iso-oncotic hemodilution, and yields an organism with stable hemodynamic conditions at rest. In these conditions, changes in oxygen-carrying capacity or other blood properties in one direction sustain or improve systemic conditions, and in the other direction cause a rapid deterioration of systemic and microvascular parameters.

This model is well suited to analyzing the effects of changes in the hemoglobin – oxygen equilibrium curve (OEC). Evaluation of the relative merits of high vs low oxygen affinity hemoglobin has been performed in comparing the high affinity blood substitute MP4 4 per cent Mal-PEG-hemoglobin, $P50 = 5.4$ mmHg (Hemospan, Sangart Inc., San Diego, CA) with the low affinity bovine-derived material PolyBvHb (10 g/dl hemoglobin, $P50 = 54$ mmHg, Biopure Inc., Boston, MA).

Figure 7.3 shows the findings regarding oxygen mass balance in the pre-microvascular and microvascular circulation using both these materials. In animals hemodiluted with MP4, 62 per cent of the total arterial oxygen content was found to be released in capillaries; hemodilution to the same degree with low affinity material was found to cause 31 per cent of the arterial oxygen content to be released in capillaries; while in animals hemodiluted with Dextran 70, 40 per cent of the arterial oxygen was found to be released in capillaries. Further analysis of these data show that MP4 releases oxygen to the capillaries *per se,* and causes more RBC-Hb bound oxygen to be released in the capillaries, while the reverse occurs in the pre-capillary circulation. Oxyglobin is a vasoconstrictor while MP4 is probably a vasodilator; therefore these findings support the concept of the inefficiency inherent to over oxygenating arterioles (Tsai *et al.*, 2004).

Changes in the oxygen equilibrium curve (OEC) can also be introduced by changing cooperativity.

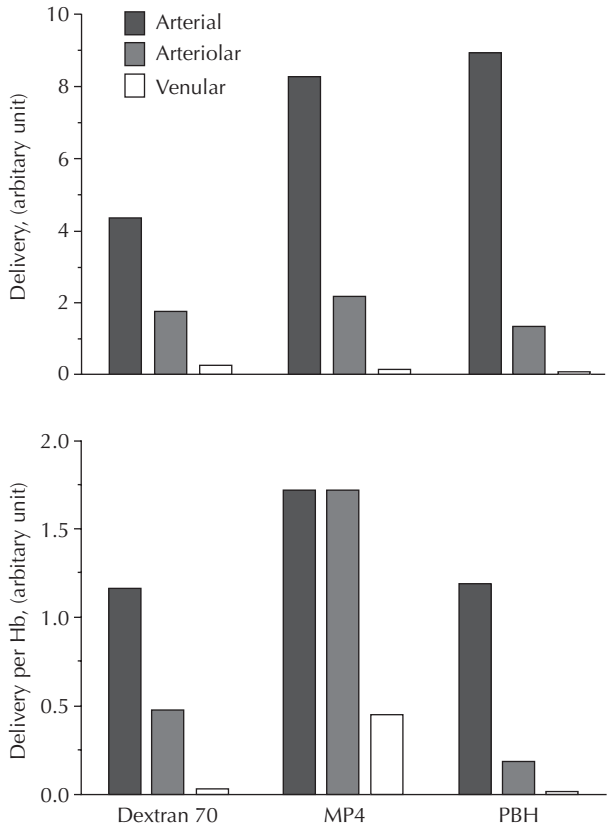

Figure 7.3 Oxygen delivery in extreme hemodilution (Hct 11 per cent) with dextran 70 kDa, 1.1 g MP4/dl and 3.7 g PolyBvHb/dl. Calculations based on measurement of cardiac output, central arterial PO_2, arteriolar PO_2 at the beginning of the microcirculation, and exit venular PO_2. PolyBvHb is a vasoconstrictor, and therefore the significantly increased total oxygen delivery capacity over extreme hemodilution with MP4 (6.7 vs 4.8 g Hb/dl for MP4) is mostly used to deliver oxygen to the arterial circulation. The relative efficacies of the two hemoglobin formulations become evident in presenting the data in terms of oxygen delivery normalized to total hemoglobin content (lower panel). Data from Tsai *et al*. (2003).

Figure 7.4 shows a similar experimental approach applied to the analysis of PEG-Hb compounds with different cooperativity obtained by modifying human hemoglobin by a process that precisely controls the number of PEG molecules reacting with hemoglobin as well as the site of conjugation, leading to a biochemically homogeneous molecular preparation (P10K2-Hb) that uses two 10-kDa PEG polymers attached to one hemoglobin molecule.

The principal finding of this study is that P10K2-Hb administered in the extreme hemodilution protocol yields a tissue PO_2 of 7.0 $\pm$ 2.5 mmHg, while MP4 with a greater number of PEG copies attached to its surface results in tissue PO_2 of 1.7 $\pm$ 0.5 mmHg. The principal differences between P10K2-Hb and MP4 are as follows:

	P10K2	MP4
Number of PEG polymers	2	5–6
Molecular weight of each PEG polymer (kDA)	10	5
Viscosity and colloid osmotic pressure (COP)	At 4 g Hb/dl concentration, 2.7 cp and 33 mmHg	At 4.2 g Hb/dl, 2.2 cp and 49 mmHg
*P*50 (mmHg)	8.3	5.4

The increased tissue PO_2 obtained with this material appears to be due to a series of incremental improvements in microvascular and macrovascular hemodynamics, comprising an increase in arteriolar and venular blood flow, an increase in FCD, and an increase in the cardiac index. Although none of the individual changes is statistically significant in relation to the others, the trend of improvement across all microvascular parameters is cumulative and causes a statistically increased FCD and tissue PO_2 (Cabrales *et al*., 2004b). Notably, although tissue PO_2 has been found to be higher with P10K2-Hb oxygen delivery, oxygen release and systemic base excess are the same for both compounds. This increased tissue PO_2 obtained in the condition of extreme hemodilution may be due to hemoglobin cooperativity, which changes the oxygen saturation of hemoglobin at very low PO_2; furthermore, in the tissue at rest the lowest *P*50 in the circulating blood appears to determine venular PO_2 and therefore tissue PO_2 when the oxygen-carrying capacity is at the verge of oxygen supply limitation.

TISSUE RESPIRATION

Tissue PO_2 is generally assumed to be representative of the level of oxygen supply to the tissue, and

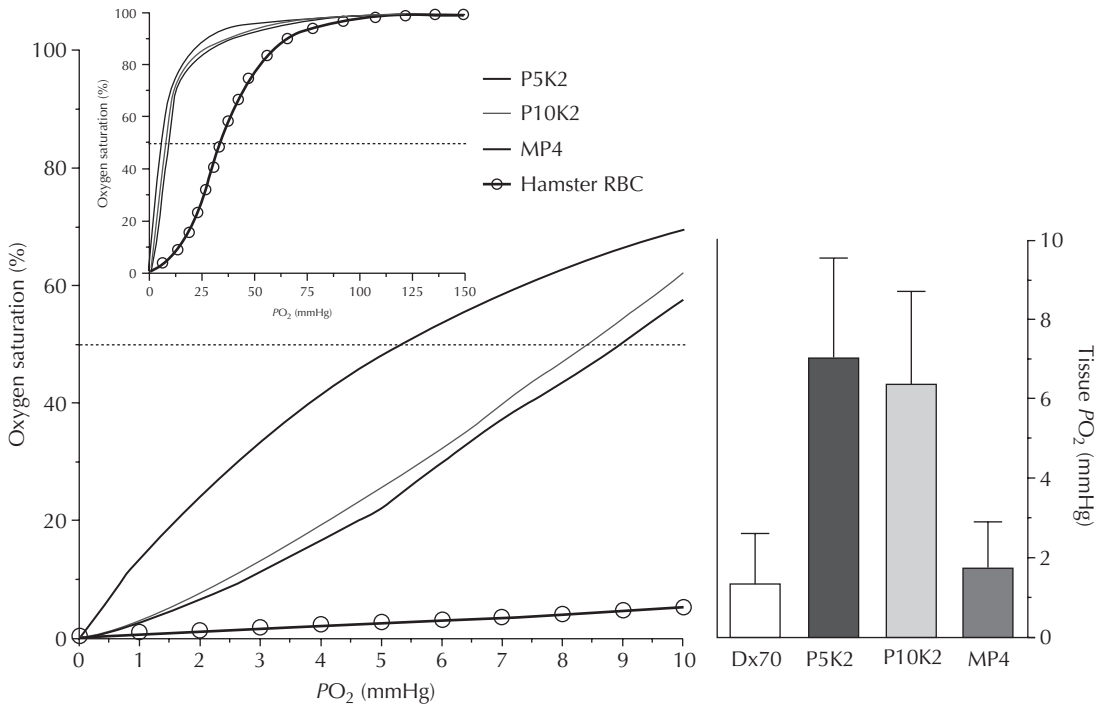

Figure 7.4 Tissue PO_2 in extreme hemodilution with PEG conjugated hemoglobins with different cooperativity. Comparison with dextran 70 kDa. P5K2-Hb, two 5-kDa polyethylene glycol polymers per Hb molecule; P10K2-Hb, two 10-kDa PEGs. MP4 (Mal-PEG-Hb, Sangart Inc., San Diego) (Cabrales *et al*., 2004b). All determinations were carried out at hematocrit 11 per cent and plasma hemoglobin ~1.1 g/dl. Cooperativity appears to cause venular and tissue PO_2 to be higher, even though total oxygen carrying capacity is the same.

this parameter is also used as a diagnostic index. However, the precise meaning of the tissue PO_2 is not clear when analyzed in terms of oxygen supply and consumption by the tissue. The results illustrated in Figure 7.4 indicate that oxygen consumption can be near normal, while tissue PO_2 can be quite different. A similar problem arises in comparing the results from the study of Tsai (2001) and Standl *et al*. (1996). The former used PolyBvHb to restore oxygen-carrying capacity in extreme hemodilution with a total blood hemoglobin concentration 6.7 g/dl (3.4 g Hb/dl in RBCs and 3.3 g Hb/dl from PolyBvHb), and found tissue PO_2 to be 0.3 mmHg in the hamster window chamber, with an increase in oxygen extraction. By contrast, the latter subjected dogs to extreme hemodilution with 6 per cent hetastarch (Hct 10 per cent), then augmented hemoglobin with Poly BvHb, and found that the mean tissue PO_2 measured with a needle electrode increased to 56 mmHg after transfusion (PO_2 in non-treated animals = 27 mmHg), and systemic oxygen extraction increased by 59 per cent.

To resolve these discrepancies Cabrales *et al*. (2004c) measured oxygen delivery and extraction in the microcirculation by red blood cells and plasma hemoglobin separately after a one-step hemodilution with PolyBvHb to a hematocrit of 19 per cent. This study showed that hemodilution with PolyBvHb abnormally lowered tissue perfusion during the initial hours of the experimental procedure to the extent that oxygen released by the microcirculation was less than half of baseline while, paradoxically, tissue PO_2 was significantly increased above normal (see Figure 7.5). Mass balance analysis showed that oxygen release into the tissue was reduced to 30 per cent of baseline, indicating that PolyBvHb caused a significant reduction of oxygen consumption. These authors also found that the wall gradient decreased significantly, indicating that vessel wall oxygen consumption was significantly depressed.

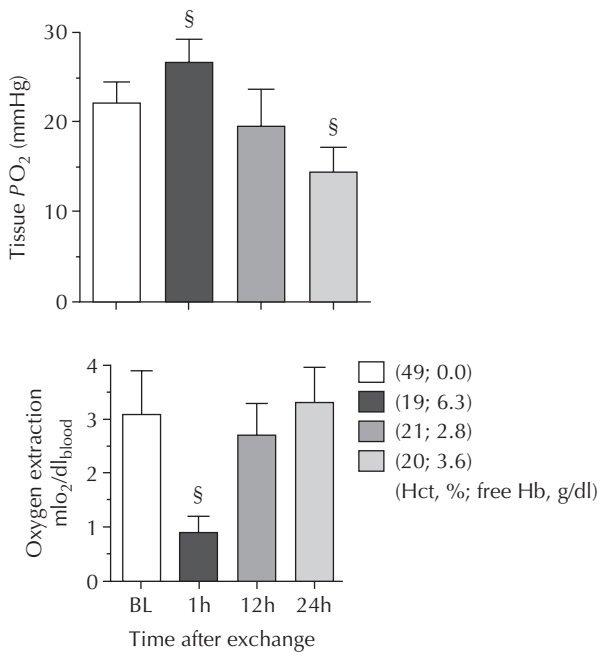

Figure 7.5 Paradoxical relationship between tissue PO_2 and oxygen extraction in the hamster window model after isovolemic blood exchange with PolyBvHb. The decrease in oxygen extraction is due to the overall decrease in oxygen consumption by the tissue, and particularly the microcirculation, indicating that PolyBvHb inhibits tissue oxygen metabolism upon administration.

Therefore, if vessel wall oxygen consumption is a significant oxygen sink, as previously discussed, its decrease makes more oxygen available to the tissue, resulting in a higher tissue PO_2.

SUMMARY

The efficacy of blood substitutes as agents for delivering oxygen to the tissue can currently be evaluated owing to the convergence of technologies that yield a quantitative assessment of oxygen transport and consumption in the microcirculation. This analysis shows that in normal conditions the oxygen and fluid transport properties of blood are matched to provide signals to the microcirculation that maintain a specific oxygen pattern which leads to an optimal partition of oxygen between the microvessels and tissue. Changing the relative magnitude of these properties affects microvascular function, shifting the localities where oxygen is unloaded, as well the mechanical condition of the microcirculation – particularly FCD. The outcome of this may be positive, increasing the efficiency of microvascular function and therefore the efficacy of blood as a transporter of oxygen; however, the converse may also occur. Present findings are that in order to optimize the efficacy of blood substitutes in maintaining microvascular function, the required changes in blood transport properties lead to unconventional sets of parameters, such as high plasma viscosity and oxygen affinity. The availability of modified hemoglobin that has no apparent toxicities suggests that new combinations of transport parameters can be devised which, recognizing that not all decreases in oxygen-carrying capacity have the same origin, may therefore be tailored for specific therapeutic interventions in diverse pathologies such as trauma, exsaguination and sepsis.

EDITOR'S SUMMARY

The primary site of oxygen supply to tissues is in capillary networks, although some oxygen is released from blood in larger vessels. Development of animal models in which PO_2 and blood flow can be studied together in individual capillaries and systems of cascading vessels of diminishing size has been critical in understanding the details of how oxygen supply to tissue is regulated. The hamster skinfold has been extremely useful in this regard, and a large number of studies have been performed using sophisticated methods to measure local PO_2, blood flow and vessel diameter. Such studies have demonstrated that while oxygen may be released in vessels with PO_2 at 20 mmHg or lower, local regulation of perfusion occurs in vessels with PO_2 in the range of 30–40 mmHg, which corresponds to the steep part of the hemoglobin oxygen equilibrium curve. Arteriolar diameter is set by the concentration of oxygen in plasma, not by hemoglobin saturation. As blood passes through progressively smaller vessels, the PO_2 drops while diameters narrow, until PO_2 reaches a minimum in capillaries. Thereafter, PO_2 begins to rise again until venous blood is mixed in the

right heart. Thus, the meaning of mixed venous PO_2 is only a crude indicator of global (total body) oxygen release, not reflecting the status of any particular organ. The oxygen concentration in actively metabolic tissue may be quite low – only a few mmHg – but mitochondria can function quite well in this environment. Thus, the meaning of tissue PO_2 may be deceptively simple, and the level may not correlate well with tissue function or overall health.

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Chapter 8

Shear Stress Mechanotransduction and the Flow Properties of Blood

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INTRODUCTION

The endothelium at the interface between solid tissue and blood is continuously subjected to mechanical stimuli/forces that arise from the motion of blood. This force generates shear stress, which is a frictional force that blood flow exerts on the endothelial surface of the vessel wall.

There is also transmural flow within the interstitial space between the endothelium and the smooth muscle cells of the arterial wall. The frictional resistance at the blood–endothelial interface determines that flow velocity is greater in midstream than at the lumen surface, and thus a gradient of velocities exists from the center of the vessel to the vessel wall. The magnitude of wall shear stress depends on the magnitude of this velocity gradient at the vessel wall. Wall shear stress for a cylindrical tube is calculated as:

$$\text{Wall shear stress} = 8\eta\, V/d \qquad (8.1)$$

where η is the fluid viscosity (poise), V is the centerline velocity (cm/s), and d is the tube diameter (cm). Wall shear stress is expressed as dynes/cm^2.

Low mean positive shear stress and oscillatory flow stimulate a cascade of cellular events that lead to endothelial cell dysfunction and atherosclerotic plaque formation (Gibson *et al.*, 1993). Conversely, high levels of mean positive shear stress have been shown to stimulate cellular responses that are essential for endothelial cell function and are atheroprotective. Endothelial cells are sensitive not only to the absolute magnitude of shear stress, but also to gradients in shear stress generated within regions of recirculating flow (White *et al.*, 2001).

Variations in mean shear stress are a direct consequence of changes in flow brought about by systemic changes in cardiac performance, local vasoactive reactions that modulate organ flow, changes in blood volume, and changes in blood viscosity arising from changes in composition and, particularly, hematocrit (blood hemoglobin content). These factors vary significantly in hemorrhagic shock, trauma and surgical interventions – i.e., all situations pertaining to the practice of transfusion medicine. The overall hypothesis of mechanochemical signal transduction is that the transfer of fluid flow forces to the cell occurs at the luminal cell surface, where the plasma membrane and associated membrane proteins such as G proteins and eNOS transduce the membrane signaling into intracellular biochemical signals. These flow-induced responses are mediated by G protein activation, as demonstrated by their inhibition by GDPS (Kuchan *et al.*, 1994), a non-hydrolyzable analog of GDP. The most elementary components necessary for mechanochemical

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signal transduction are a phospholipid bilayer and heterotrimeric G proteins. It was recently demonstrated that the physical properties of the endothelial plasma membrane itself are changed under flow (Haidekker *et al.*, 2000, 2001), leading to the hypothesis that membrane associated proteins, such as G proteins, are activated by increased membrane fluidity.

SHEAR STRESS AND THE RELEASE OF VASOACTIVE MEDIATORS

Furchgott and Zawadzki (1980) demonstrated that relaxation of vascular smooth muscle following acetylcholine administration was dependent on the integrity of the endothelium, which responded by upregulating the production of the free radical gas nitric oxide (NO), which was subsequently identified as the endothelium-derived smooth muscle relaxing factor. In the endothelium the isoform of nitric oxide synthase (eNOS) converts the amino acid L-arginine to L-citrulline and nitric oxide, shear stress being the most potent and direct regulatory factor of eNOS activity (Kuchan and Frangos, 1994) and gene expression (Noris *et al.*, 1995), while low levels of shear stress and turbulent flow have the opposite effect. The sudden onset of flow induces a burst of nitric oxide production. This process is both calcium- and G protein-dependent. In contrast, steady shear stress induces a sustained release of nitric oxide that is calcium and G protein-independent (Kuchan and Frangos, 1994). Transient production of nitric oxide is directly related to the rate of change in shear stress rather than to its absolute magnitude, whereas the sustained release of nitric oxide is directly related to the level of mean positive shear stress (Kuchan and Frangos, 1994).

Shear stress also induces the release of prostacyclin (PGI2) from the endothelium. PGI2 is an endothelium-derived vasodilator that relaxes the underlying vascular smooth muscle through the activation of adenylate cyclase and the subsequent initiation of a cAMP signaling cascade. The mechanotransduction mediated release of PGI2 is particularly important in the development of blood substitutes based on molecular hemoglobin because while nitric oxide is scavenged by molecular hemoglobin, PGI2 is not. Furthermore, PGI2 was the first inhibitor of platelet aggregation shown to be released from endothelial cells by exposure to shear stress (Frangos *et al.*, 1985; Grabowski *et al.*, 1985). As in the case of nitric oxide, the release of prostacyclin from endothelial cell cultures is enhanced when the flow pattern is pulsatile versus steady (Frangos *et al.*, 1985). Flow-induced release of PGI2 is biphasic. After an initial rapid release, production slowly declines over several hours before recovering to maintain a steady release rate (Berthiaume and Frangos, 1994). The first phase of rapid release is tightly linked to calcium mobilization. *In vitro*, production of flow-induced PGI2 is significantly inhibited when cultured endothelium cells are exposed to an inhibitor of intracellular Ca^{2+} mobilization (Bhagyalakshmi and Frangos, 1989). The second phase is directly related to the magnitude of shear stress, and an exogenous source of the arachidonic acid (a precursor to PGI2 synthesis).

MECHANOTRANSDUCTION AND MECHANOSENSORS

A number of endothelial mechanosensors have been proposed, including caveolin/caveolae, integrins, the cytoskeleton, PECAM-1 (CD31), the phospholipid membrane and the glycocalyx. However, the primary mechanosensor which transduces this mechanical stimulus into a biochemical signal remains unknown (Davies, 1995). Caveolin has been implicated as the endothelial mechanosensor for the activation of caveolin-associated MAP kinase and eNOS in a rat lung perfusion model (Oh and Schnitzer, 2001). These studies, however, are inconclusive, since there was no clear differentiation between stimuli due to shear stress or stretch, or indication of whether mechanotransduction in the pulmonary capillaries is similar to that in arterial vessels. Several studies support $\alpha 1$ and $\alpha v \beta 3$ integrin-mediated mechanotransduction in endothelial cells (Shyy and Chien, 2002). Two methods have been used to support this conclusion. The first involves the use of passaged human umbilical vein endothelial cells (HUVEC) plated for just 2 hours onto surfaces coated with ECM, and immediately subjected to step shear stress. When plated on fibronectin or vitronectin the cells were mechanoresponsive, while those on laminin or collagen were not (Jalali *et al.*, 2001). The second method involved the treatment of endothelial cell with antibodies against specific integrins (Liu *et al.*, 2002). In both cases it is likely that PECAM-1 homophilic interactions at the cell–cell junctions in addition

to the integrin–ECM interactions were disrupted. Thus it is still unclear what role, if any, integrins have in endothelial mechanotransduction. Disruption of the cytoskeleton has been proposed to impair flow-induced responses; however, Knudsen and Frangos (1997) showed that cytoskeletal disruption enhanced shear-induced nitric oxide release is mostly due to the enhanced membrane fluidity caused by the uncoupling of the cytoskeleton from the membrane. Furthermore, Gudi *et al*. (1998) demonstrated that mechanochemical transduction can occur in defined liposomes containing G proteins and phospholipids, and in the absence of integrins and cytoskeleton. While it is clear that the cytoskeleton may modify the mechanochemical transduction process, to date there has been no evidence directly demonstrating its role as the mechanosensor.

Fujiwara proposed that the platelet endothelial cell adhesion molecule (PECAM-1) at the cell–cell adhesion site is the mechanoresponsive molecule (Fujiwara *et al*., 2001). PECAM-1 is a 130-kD glycoprotein member of the immunoglobulin family of cell adhesion molecules. It is constitutively expressed on all vessel types, and is localized to cell–cell contacts and to apical, lumen-facing cell areas. PECAM-1 mediates cell adhesion and has signaling capabilities (Newman and Newman, 2003). Fujiwara's group demonstrated that PECAM-1 is tyrosine phosphorylated at one of its two ITIM domains within 30 s by fluid shear stress, or hyper- and hypo-osmotic shock. In a guinea pig model with surgical coarctation in the aorta, PECAM-1 phosphorylation was prominent in endothelial cells lining the high shear region (Kano *et al*., 2000). Once phosphorylated the ITIM domain is the binding site for SHP-2, which when activated leads to activation of ERK 1/2. The tyrosine kinase involved appears to be independent of increases in calcium, activation of protein kinase C, membrane hyperpolarization, and stretch-activated channels. These are all necessary attributes of the mechanosensor molecule, which must lie upstream of all other signaling.

THE ROLE OF THE GLYCOCALYX

The endothelial glycocalyx is an organized mesh on the luminal endothelial cell surface, consisting of proteoglycans, glycosaminoglycans and associated plasma proteins such as albumin (Vink *et al*., 1995). This endothelial layer functions as a protective barrier between endothelial cells and flowing blood by contributing to the endothelial permeability barrier (Vink *et al*., 2000) and modulating leukocyte interactions with the endothelium (Mulivor and Lipowsky, 2004), and has been proposed to be a mechanosensor (Mulivor and Lipowsky, 2002).

The glycocalyx has been implicated as a mechanosensor structure (Pohl *et al*., 1991), and a recent report appears to support that concept. Mochizuki *et al*. (2003) have shown that hyaluronidase-induced degradation of hyaluronic acid glycosaminoglycans in canine excised vessels inhibited flow-induced nitric oxide release. The specific direction of this interaction is not clear, since studies on canine coronary flow lead to the conclusion that 'weakening the integrity of glycocalyx matrix (by hyaluronidase) allows an enhanced transmission of shear forces of flowing blood to the endothelial cells' (Spaan *et al*., 2001), that 'hyaluronidase treatment of the endothelial glycocalyx increases flow-dependent dilatation' and that 'this may be due to enhanced shear-dependent endothelial NO release' (Dekker *et al*., 2001). Thus the role of the glycocalyx in modifying the shear stress response in endothelial cells is not clear; however, the glycocalyx hypothesis of mechanotransduction is particularly important in the analysis of the effects of altered blood fluid properties because it is a system of molecules that is located at the interface between blood and the endothelial surface that contains the mechanosensory transducers.

Tarbell and coworkers (Florian *et al*., 2003) showed that enzymatic removal of heparan sulfate (the dominant glycosaminoglycan of the endothelial glycocalyx) with heparinase completely inhibited shear-induced nitric oxide production. There is a growing body of evidence showing that the steady state glycocalyx dimension is the result of synthesis and degradation of its constituents (Mori *et al*., 1999). It was recently demonstrated that ischemia (Mori *et al*., 1999) and oxidative stress (Vink and Duling, 2000) reduce the endothelial glycocalyx by a mechanism involving increased shedding, resulting in increased permeability and adhesiveness of blood cells to the endothelial membrane. Most circumstances where blood substitutes are administered involve some degree of ischemia. Together with the evidence that heme-containing blood substitutes induce oxidative stress (Alayash, 2004), it is possible that treatment with such materials can

induce a reduction in the thickness of the glycocalyx and the subsequent alteration of flow-induced mechanotransduction.

CHANGES OF SHEAR STRESS IN THE MICROCIRCULATION

The precise level of shear stress in the microcirculation can only be approximately determined because of the disparity of vessel size and flow velocities, and the uncertainty of the value of local viscosity since hematocrit in the microcirculation decreases as a function of blood vessel size, requiring the correction developed by Lipowsky and Firrell (1986) for estimating local blood viscosity on the basis of systemic samples.

One of the first well-defined differences in microvascular function determined by wall shear stress was obtained in the study by Tsai *et al*. (1998), who performed hemodilution to the critical level of oxygen supply limitation with plasma viscosities of 2.2 cp and 1.4 cp. The central hematocrit in this study was 12 per cent, a condition that was shown by Lipowsky and Firrell (1986) to produce a similar microcirculatory hematocrit. This study showed that under otherwise identical conditions of intrinsic blood oxygen-carrying capacity, with plasma and therefore blood viscosity as the only variable, microvascular function was fundamentally different between groups. The high-viscosity group had near normal functional capillary density and increased microvascular flow, while the opposite resulted with low-viscosity perfusion.

Wall shear rate was estimated using the mean diameter and velocity, and averaged 600 s in arterioles and 127 s in venules, leading to a wall shear stress of 22.8 and 4.5 dynes/cm^2 respectively. The extreme hemodilution protocol reduced the shear stress in the high-viscosity group to 13.7 and 4.8 dynes/cm^2, while the low-viscosity group had shear stress of 8.6 and 1.7 dynes/cm^2. The results of this study showed that there was a significant difference in shear stress caused by the different plasma viscosities, and that a two-fold decrease for the change in shear stress may be a threshold for causing changes in microvascular function.

Increase in vessel wall shear stress by elevation of plasma viscosity using high molecular weight dextran 500 has been shown to induce sustained NO-mediated dilation in the hamster cremaster muscle *in vivo* (de Wit *et al*., 1997) where increasing the plasma viscosity by 64.3 per cent led to a 24.3 per cent dilation in large feeding arterioles. Systemic vasodilatation, implying a reduction in total peripheral resistance during moderate hemodilution, has also been shown to be a result of endogenous nitric oxide release (Doss *et al*., 1995). Microvascular shear stress for oxygen-carrying and non-oxygen-carrying blood substitutes are shown in Figure 8.1 for conditions in which hematocrit was reduced to 11 per cent (extreme hemodilution).

Increasing plasma viscosity may be beneficial for tissue perfusion, provided that overall viscosity-dependent vascular resistance does not increase to pathological levels. The basis for this phenomenon is that vascular resistance increases linearly with blood viscosity, while small changes in vascular diameter have a disproportionate effect – as shown by Hagen-Poiseuille's equation, which defines the flow Q in tube as:

$$Q = \pi R^4 (8\eta)^{-1} \Delta P/\Delta L \qquad (8.2)$$

where R is the tube radius, η is the fluid viscosity and $\Delta P/\Delta L$ is the local longitudinal pressure gradient. Previous findings support this concept. Comparison of tissue oxygenation achieved during moderate hemodilution with 3 per cent and 6 per cent dextran 60, a 33 per cent difference in plasma viscosity, showed similar elevations in PO_2 levels on the surface of liver and skeletal muscle (Gustafsson *et al*., 1981). Investigators found that a higher-viscosity replacement fluid was able to achieve increased organ blood flow not observed with lower-viscosity fluid. High-molecular dextran 500 solutions have also been proposed to lower mortality and limit the severity of pancreatitis (Schmidt *et al*., 1993). Waschke *et al*. (1994) found that cerebral perfusion was not compromised when they increased plasma viscosity while keeping the oxygen content of blood constant. They achieved this by performing a complete blood exchange with an exchange fluid consisting of cell-free hemoglobin and 2 per cent polyvinyl-pyrrolidone (400 000 MW). Chen *et al*. (1989) exchanged whole blood with a 20 per cent w/v solution of dextran 500 in blood, elevating plasma viscosity four-fold (4 cp), and observed compensatory vasodilation reflected by a reduction of vascular hindrance in several vital organs.

The functional significance of the level of shear stress is evidenced by the evaluation of functional capillary density present in the tissue as a function

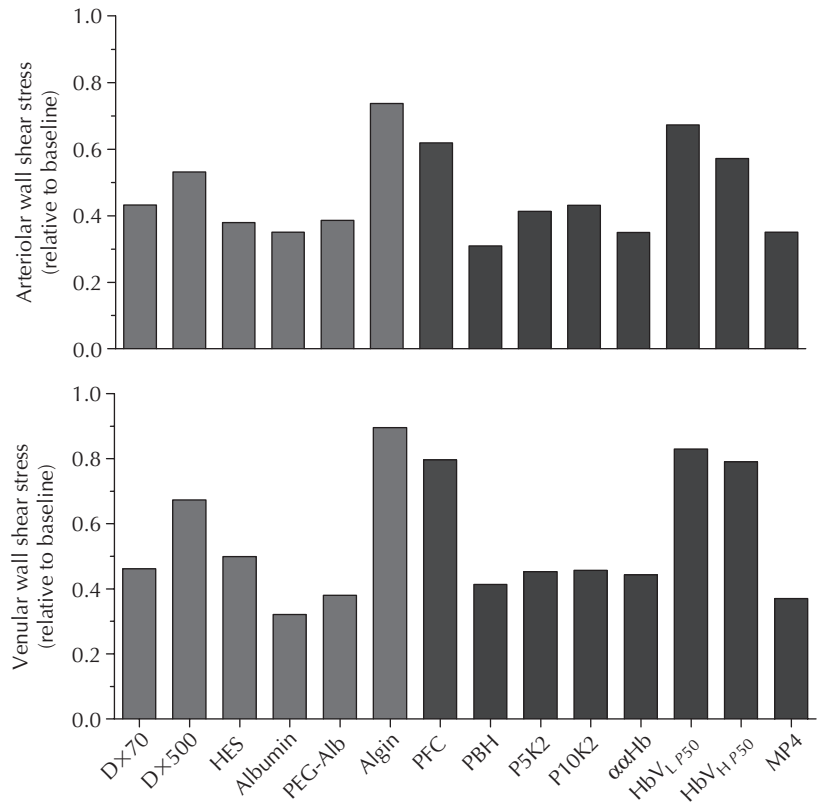

Figure 8.1 Arteriolar and venular wall shear stress relative to control conditions (1.0) in the same microvessels in the hamster window model. All tests consisted of first lowering hematocrit in two steps, to 20 per cent hematocrit using 6 per cent dextran 70 kDa (Dx70), and then to 11–12 per cent using the different test materials. The rationale for this experiment is that 11–12 per cent Hct is the point at which the circulation is on the verge of oxygen supply limitation for this species. The materials tested were 6 per cent dextran 500 kDa (Dx 500), Pharmacia (now Pfizer Inc.); 10 per cent hydroxyethyl starch 200 kDa (HES); 5* human serum albumin (Albumin); 4 per cent polyethylene glycol conjugated human albumin (PEG-Alb); 0.7 per cent alginate (Algin), FMC Biopolymer, Brakrøya, Norway; 30 per cent perfluorocarbon (PFC), Oxycite, Synthetic blood International, Costa Mesa, CA; 13.2 per cent polymerized bovine hemoglobin (PBH), Oxyglobin, Biopure Inc., Boston, MA; 4 per cent conservatively pegylated hemoglobin, two polymers of either 5 kDa or 10 kDa (P5K2 and P10K2) (Cabrales *et al*., 2004); 8 per cent αα-crosslinked hemoglobin (ααHb) prepared according to LAIR (Letterman Army Institute of Research, San Francisco, CA); hemoglobin vesicles with *P*50 9 mmHg (HbV_{LP50}) and 29 mmHg (HbV_{HP50}), ARISE, Waseda University, Tokyo, Japan; and MP4 (4 per cent Mal-PEG-Hb, Sangart Inc., San Diego, CA).

of this parameter, shown in Figure 8.2. Functional capillary density is the number of capillaries per unit volume of tissue through which there is blood flow, and has been shown to be a critical parameter in evaluating efficacy of blood replacement fluids at the level of the microcirculation (see Chapter 7).

EFFECTS IN THE PLASMA LAYER

The plasma layer comprises the compartment in the vicinity of the blood vessel wall. In this region the concentration of red blood cells changes progressively from that in the core to virtually zero at the vessel wall boundary during flow conditions. This rarefaction of red blood cell concentration occurs because of geometric and hydrodynamic effects. For red blood cells to be evenly distributed across the cross-section of blood vessels, the vessel wall surface would have to coincide with the statistical average of the geometrical center of red blood cells. Since this is impossible, the concentration of red blood cells decreases beyond a distance that is approximately equal to the average geometrical

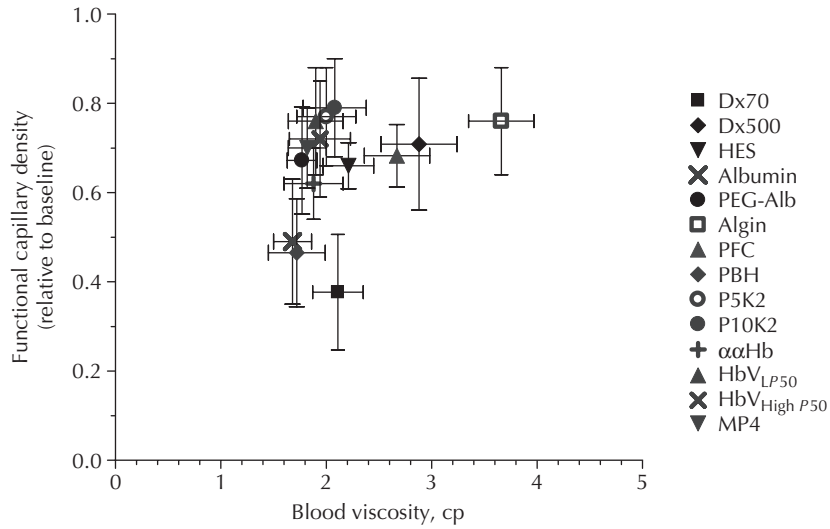

Figure 8.2 Functional capillary density (FCD) as a function of blood viscosity measured systemically. Extreme hemodilution (Hct 11–12 per cent). The materials tested were 6 per cent dextran 500 kDa (Dx 500), Pharmacia (now Pfizer Inc.); 10 per cent hydroxyethyl starch 200 kDa (HES); 5* human serum albumin (Albumin); 4 per cent polyethylene glycol conjugated human albumin (PEG-Alb); 0.7 per cent alginate (Algin), FMC Biopolymer, Brakrøya, Norway; 30 per cent perfluorocarbon (PFC), Oxycite, Synthetic blood International, Costa Mesa, CA; 13.2 per cent polymerized bovine hemoglobin (PBH), Oxyglobin, Biopure Inc., Boston, MA; 4 per cent conservatively pegylated hemoglobin, two polymers of either 5 kDa or 10 kDa (P5K2 and P10K2) (Cabrales *et al*., 2004); 8 per cent αα-crosslinked hemoglobin (ααHb) prepared according to LAIR (Letterman Army Institute of Research, San Francisco, CA); hemoglobin vesicles with *P*50 9 mmHg (HbV_{LP50}) and 29 mmHg (HbV_{HP50}), ARISE, Waseda University, Tokyo, Japan; and MP4 (4 per cent Mal-PEG-Hb, Sangart Inc., San Diego, CA). High-viscosity blood attained with plasma expanders yields the same FCD as low-viscosity blood with pegylated hemoglobin in plasma, suggesting that the PEG materials may increase shear stress by interacting with the glycocalyx and lowering the extent of the plasma layer.

radius of the red blood cells. Considering the discoid shape of red blood cells, this distance may be assumed to be the average of the RBC radius (~4.2 μm) and half the thickness (or 1.2 μm), or about 2.7 μm, giving rise to the so-called 'wall exclusion effect' (Maude and Whitmore, 1956).

Experimental model studies that began with Goldsmith and Mason in 1961 showed that suspensions of particles flowing in tubes are subjected to hydrodynamic effects that cause the migration of particles towards the tube centerline. The geometric constraint and hydrodynamic phenomena are considered to be the cause of the Fahraeus effect, by which the flow of a suspension in branching networks, like blood in the microcirculation, decreases particle concentration in side branches. The plasma layer is visually evident in intravital studies of blood flow in the microcirculation; however, this space could also be occupied in part by a low-density molecular species whose optical properties are not too different from those of plasma. *In vivo* measurements of capillary tube hematocrit versus discharge hematocrit in the microcirculation were found not to be in agreement, leading Klitzman and Duling (1979) to postulate the existence of a deformable protein layer that lines the microvessels, whose thickness may be as much as 1 μm (Secomb and Pries, 2001). This deformable protein layer is therefore an additional component defining the extent of the plasma layer.

BLOOD SUBSTITUTES AND MECHANOTRANSDUCTION

Mechanotransduction in the endothelium is a fundamental mechanism that controls the production of a vast repertoire of mediators. The mechanical origin of this process is centered on the shear stress developed at the surface of the endothelium. A critical aspect of this process is the extent of the plasma layer where vessel wall shear stress develops, and the viscosity of the fluid in this layer. This plasma layer has already been

identified to be critical for the process of facilitated diffusion in the presence of molecular hemoglobin (McCarthy *et al.*, 2001). Considering that the trend in the development of blood substitutes leads to the formulation of large-volume molecular species with unusual surface molecular properties, such as PEG-hemoglobin, it is likely that their effects may be also due to the interaction with parameters that determine the extent of the plasma layer.

The introduction of blood substitutes into the circulation may produce significant effects on the development and maintenance of shear stress in the microcirculation. Substitution of the oxygen-carrying capacity of red blood cells by molecular hemoglobin lowers blood viscosity and shear rate in the plasma layer because of the dilution of the red blood cell mass and return of the blunt velocity profile to the theoretical parabolic profile (Schmid-Schoenbein and Zweifach, 1975; Briceño *et al.*, 2004). Conversely, vasoconstriction due to nitric oxide scavenging may increase shear stress due the effect of diameter. Extrusion of the large molecular species or their aggregates into the plasma layer may increase viscosity and interaction with the glycocalyx. Furthermore, consideration should be given to whether the pathology being remedied may have affected the glycocalyx *per se*.

EDITOR'S SUMMARY

Recent studies have established important links between local shear forces in the microcirculation and the release of vasoactive mediators such as nitric oxide and prostaglandins. These mechanisms provide a new understanding of how plasma viscosity might be related to the regulation of blood flow, particularly local blood flow in the microcirculation. Thus when blood is lost, as in hemorrhage, hemodilution lowers plasma viscosity as well as the shear forces exerted on endothelium. This in turn reduces the release of nitric oxide, producing local vasoconstriction. As a normal mechanism this would conserve central blood volume in shock, but would reduce local tissue perfusion. With this new information in mind, cell-free oxygen carriers must be designed with their effects on plasma viscosity and the implications for mechanotransduction kept in mind.

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Chapter 9

Local Regulation of Blood Flow

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INTRODUCTION

On a moment-by-moment basis, oxygen delivery to the tissue is the most important function of the circulatory system. Oxygen stores in the tissue are limited, and are quickly exhausted when blood flow is interrupted. In the absence of oxygen, organ function is dependent on the ability of the tissue to obtain energy from anaerobic metabolism. This ability varies from organ to organ, but in most cases tissue can sustain only limited function. This limitation is especially pronounced for the brain and myocardium, where oxygen consumption is high and oxidative metabolism provides the only effective means of sustaining normal function. Therefore it is not surprising that robust local regulatory mechanisms provide increased blood flow when energy requirements in an organ increase.

While recognizing the primacy of oxygen delivery to the tissue, it is important to note that the circulatory system also serves other important functions without which a normal *milieu interne* for the tissues could not be maintained and the organism would not long survive. These functions include removal of the waste products of metabolism, maintenance of normal hydration of the tissues, transport of substances between organs (such as from the intestine to the liver), provision of a first line of defense against invading organisms, and maintenance of a communication network for the endocrine system – to list but a few. Given these diverse functions, it should also not be surprising that the local mechanisms for regulation of blood flow include several that are not directed primarily toward oxygen delivery to the tissues.

The importance of local flow regulatory mechanisms tied to oxygen delivery versus other functions varies from organ to organ. Some appreciation of this fact may be seen in the relation between blood flow and oxygen delivery when the organism is in the rest state, as shown in Figure 9.1. As shown in that figure, venous oxygen content varies among organs, indicating that the fraction of cardiac output delivered to a particular organ is not determined solely by the oxygen requirements of the organ. In the case of the kidney, the fraction of oxygen consumed is only 10 per cent of the oxygen delivered, as calculated from the arterial-venous oxygen content difference and the arterial oxygen content. At the other extreme, the myocardium extracts 65 per cent of the oxygen delivered and is closer to the brink of hypoxia. However, available evidence indicates that the level of blood flow in myocardium when the individual is in the resting condition is not determined by metabolic demand alone.

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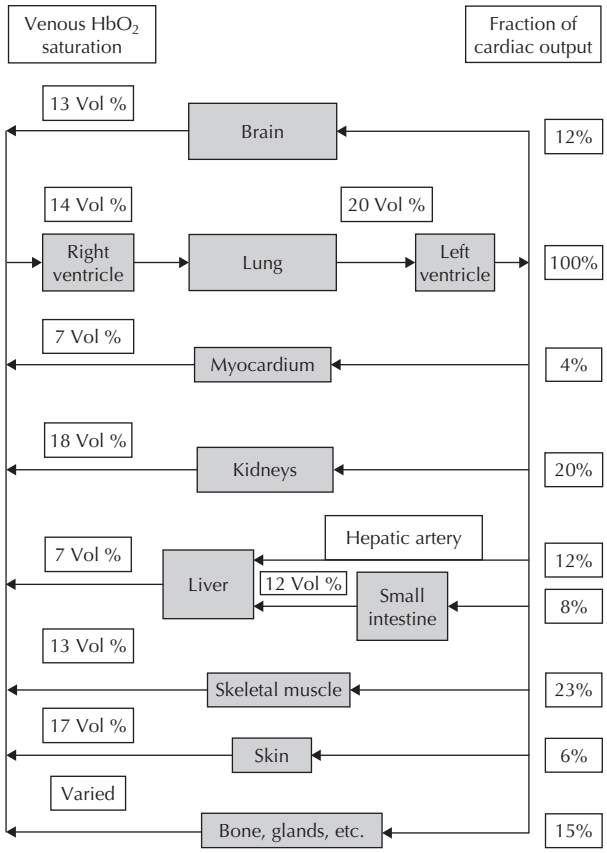

Figure 9.1 Distribution of cardiac output to the peripheral circulation and blood oxygen content in the venous blood draining major organs in the resting state.

TISSUE OXYGEN SUPPLY AND DEMAND

Under normal circumstances oxygen delivery to various organs is more than adequate to meet the requirements for oxidative metabolism. Measurements in microcirculatory preparations (primarily muscle) from a number of laboratories indicate that PO_2 in the interstitial fluid of resting tissue is generally between 20 and 30 mmHg (Tsai *et al.*, 2003). These measurements include tissue regions at the venous end of the capillary network, where the oxygen tension is expected to be lowest. By contrast, the critical PO_2 in interstitial fluid for a shift to anaerobic metabolism is 3 mmHg or less (Richmond *et al.*, 1999). Additional evidence that no tissue areas in resting muscle are on the verge of hypoxia is provided by studies in which complete stoppage of blood flow leads to a shift to anaerobic metabolism in the tissues only after a delay of 10–45 s in rat and cat skeletal muscle respectively (Toth *et al.*, 1996; Richmond *et al.*, 1999). While maintaining tissue PO_2 well above the minimal requirements for oxidative metabolism, blood flow regulatory mechanisms also appear to provide an upper limit on oxygen levels in the tissues. This may have a protective effect on the tissues, since the toxic effects of oxygen through formation of reactive oxygen species (ROS) are well known.

In considering the mechanisms responsible for blood flow regulation and oxygen delivery, a clear distinction needs to be made between conditions that apply when the organism is at rest as described above and those that obtain under circumstances of increased oxygen demand. The dominant mechanisms of flow regulation are different in the two circumstances. When metabolic demand increases, specific feedback mechanisms come into play. A classical example of how blood flow is determined by demand is the increase in blood flow in working skeletal muscle, or functional hyperemia. As shown in Figure 9.2, the flow increase appears to be a linear function of oxygen consumption of the muscle. However, initially the increased oxygen requirement of the tissue is met in part by extracting additional oxygen from the blood, as is evident in the decrease in PO_2 and oxygen saturation of the venous blood. Observations such as this have led to the supposition that a reduction in tissue PO_2 leads to metabolic changes and production of vasodilator metabolites. The evidence for mechanisms in which tissue PO_2 is linked to vascular tone is considered further below.

A second circumstance in which an organ faces a possible shortfall in oxygen delivery relative to consumption is during reduction of arterial pressure. As shown in Figure 9.3, steady state blood flow in the coronaries and skeletal muscle is well regulated in the arterial pressure range 60–160 mmHg. If the normal arterial pressure is 100 mmHg, for example, reduction or increase of arterial pressure as shown for skeletal muscle will lead to vasodilation or vasoconstriction respectively, and a return of blood flow to its initial value. This response pattern, autoregulation of blood flow, is seen in virtually all organs of the body.

While the autoregulatory response in other organs is similar that to Figure 9.3, the causal mechanisms are not necessarily the same. One causal mechanism is the myogenic response or pressure sensitivity of the arterioles. This response causes constriction when internal pressure is

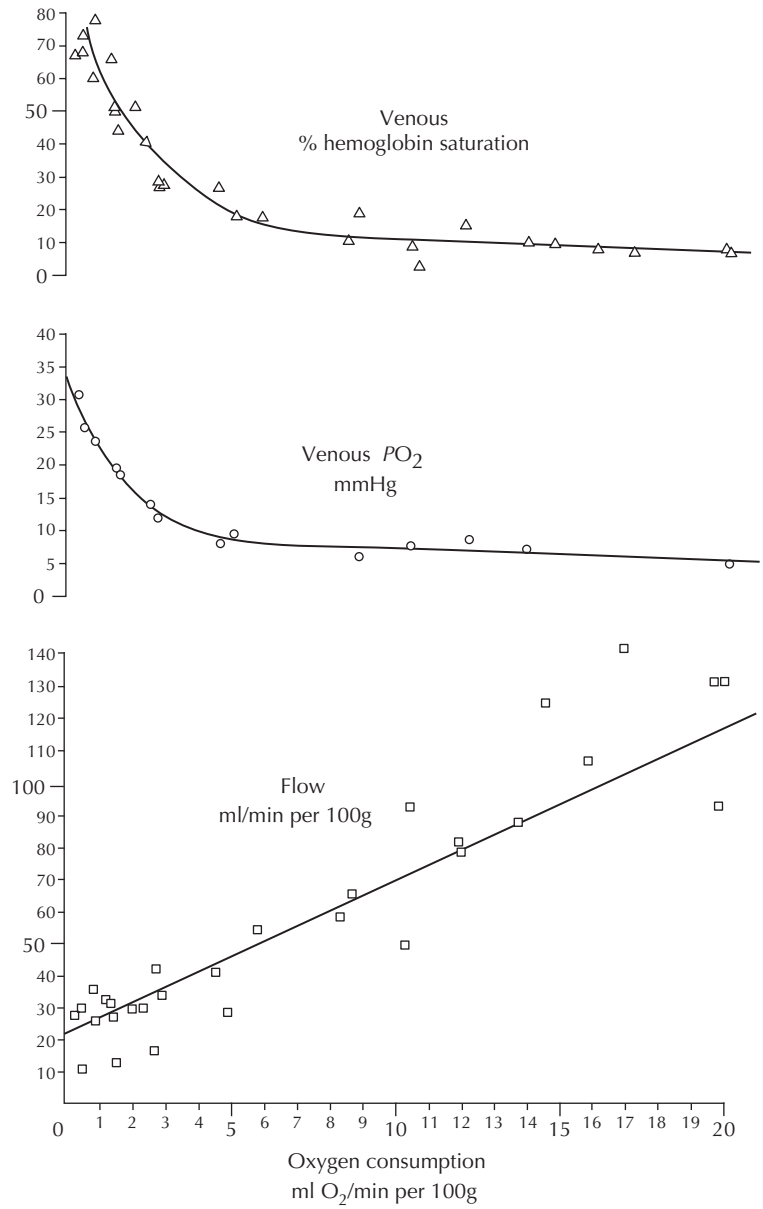

Figure 9.2 Relation between blood flow and oxygen consumption in contracting muscle, together with oxygen content and PO_2 of venous blood (from Sparks, 1978, with permission).

elevated, and dilation when pressure is reduced. It has been shown by means of a simple model that this mechanism is capable of providing an essentially constant flow over a considerable range of arterial pressure (Oien and Aukland, 1983). This mechanism acts in such a manner that capillary pressure is also held constant, and the autoregulation of flow may be incidental to that function (Johnson, 1986). In the brain this mechanism may also be important in preventing undue elevation of pressure in the capillary bed with sudden increases in arterial pressure. Since the myogenic response is a normal component of arteriolar vascular tone, it may contribute to autoregulation generally in the body. Mechanisms directly linked to flow may also be important since when arterial pressure is reduced, blood flow would fall, as would PO_2 in the tissue and secondarily in the arterioles. If the fall in tissue PO_2 is pronounced, it could induce a metabolic shift

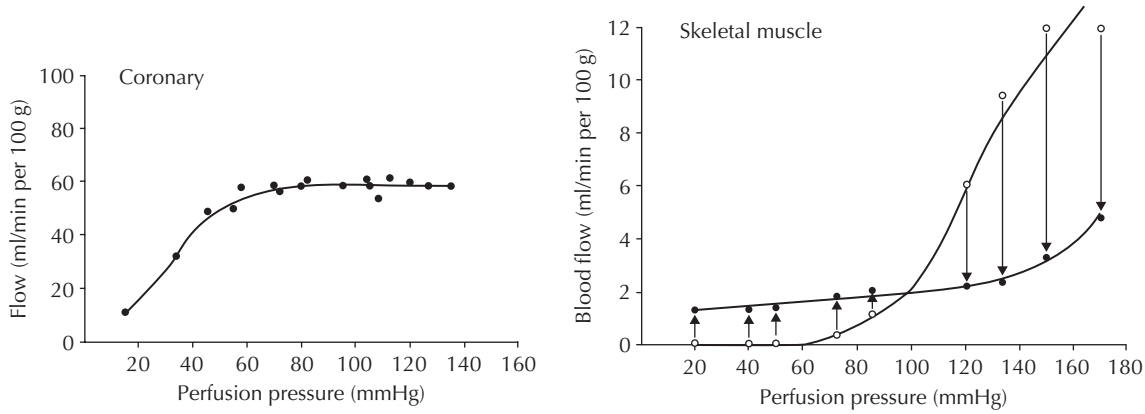

Figure 9.3 Examples of blood flow autoregulation in coronary circulation and skeletal muscle. Steady state values only are shown for the coronary circulation. For skeletal muscle, the effect on flow is shown when arterial pressure is rapidly raised or lowered from a control level of 100 mmHg in various increments in the range 20 mmHg to 170 mmHg and held at the new level until flow stabilizes. The initial flow (open circles) shows the passive behavior of the vascular bed with a sudden pressure change, and steady state flow (closed circles) shows the effect of the autoregulatory mechanism which tends to return flow to a constant level. The length of the vertical lines indicates the magnitude of the autoregulatory response at a given pressure. (Modified from Johnson, 1980, with permission.)

and production of vasodilator metabolites. In organs such as the brain, reduction of arterial pressure leads to a relaxation of myogenic tone, increased levels of adenosine (a potent vasodilator), neural mechanisms and activation of K^+ channels (Faraci and Heistad, 1998). In the kidney, tubulo-glomerular feedback is a specialized mechanism involved in maintenance of glomerular filtration rate through a vasoconstrictor effect on the pre-glomerular vessels (Inscho *et al*., 2004), and contributes together with the myogenic mechanism to the very precise autoregulation of blood flow seen in that organ.

A third condition in which oxygen supply may not meet demand is reactive hyperemia. This is somewhat related to autoregulation of blood flow, in which blood flow is elevated after a period of flow arrest. It has been shown that with brief periods of occlusion, in which the oxygen requirements of the tissue are met by oxygen stores in the tissue and blood, the hyperemia involves relaxation of the myogenic response (Johnson *et al*., 1976). More recently, the initial dilation has also been shown to be due to formation of nitric oxide in the endothelium due to deformation of the endothelial cells with collapse of the arterial vessels (Koller and Bagi, 2002). The hyperemic response to brief occlusions is characterized by a fixed duration of hyperemia and increased peak flow as the occlusion duration increases. When the period of hyperemia is sufficient to exhaust the oxygen stores a shift to anaerobic metabolism in the parenchymal cells occurs, leading to production of vasodilator metabolites. In this case the hyperemia duration increases in proportion to the magnitude and duration of the rise in tissue NADH, which is an indicator of the anaerobic state (unpublished observations, A. Toth, M. Pal, M. Intaglietta and P. C. Johnson).

A fourth condition in which oxygen delivery to the tissue is reduced relative to consumption is hypoxic hypoxia, in which the oxygen level of the arterial blood is reduced – as happens, for example, at high altitude. In this circumstance both the arterial and venous oxygen levels decrease. The ability of the organ to maintain normal oxygen consumption depends importantly on the vascular response to hypoxia. An example in which oxygen consumption is maintained is shown in Figure 9.4, from a study by Ray *et al*. (2002) utilizing the rat hind limb. In this study, inspired gas oxygen concentration was reduced to 8 per cent for 5 minutes. Femoral vascular conductance doubled while arterial pressure decreased and blood flow (not shown) did not change significantly due to the simultaneous drop in arterial pressure. Blockade of prostaglandin synthesis significantly reduced the increase in vascular conductance, as did subsequent blockade of

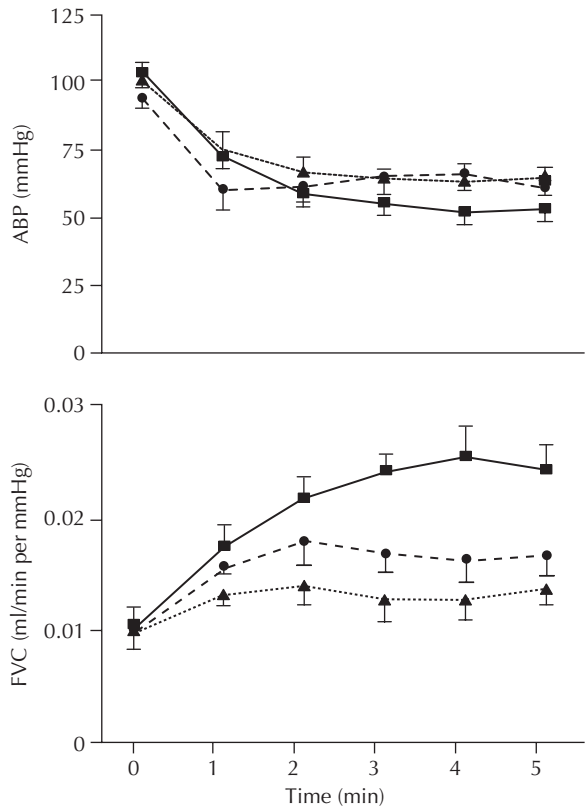

Figure 9.4 Change in peripheral vascular conductance (PVC) in the rat hind limb and arterial blood pressure (ABP) during 5-minute exposure to 8% oxygen in inspired gas. Effect of prostanoid blockade is shown as dashed line and subsequent adenosine blockade as dotted line. (Modified from Ray *et al.*, 2002, with permission.)

adenosine receptors – specifically A1 receptors. Since oxygen consumption of the preparation did not change, a shift to anaerobic metabolism of the parenchyma is not involved in the increase in vascular conductance.

Oxygen delivery to the tissue may also be reduced by anemia, in which case the partial pressure of oxygen in the arterial blood is normal but the oxygen-carrying capacity is decreased due to lower red cell content. In addition, whole blood viscosity is reduced, which lowers resistance to blood flow and would be expected to increase flow and compensate to a degree for the lower oxygen content. However, reducing blood viscosity is not without its limitations, since it would decrease wall shear stress in the arterial vessels and decrease production of nitric oxide by the endothelium, which would lead to vasoconstriction.

CELLULAR METABOLIC FEEDBACK

A longstanding hypothesis to explain the coupling of oxygen delivery to oxygen demand is that tissue concentration of diffusible vasodilator products of aerobic metabolism, such as carbon dioxide, is dependent on the ratio of blood flow to oxygen consumption. The brain circulation is very responsive to changes in carbon dioxide (Faraci and Heistad, 1998), and there is evidence that it may contribute to autoregulation in the coronary circulation (Broton and Fiegl, 1992). In other vascular beds such as skeletal muscle, however, vascular tone is not highly sensitive to carbon dioxide levels (Faraci and Heistad, 1998). Another venerable hypothesis is that a shift in the ratio of delivery to demand causes PO_2 in some tissue areas, most likely at the venous end of the capillary network, to fall below critical values, causing a shift from oxidative metabolism to anaerobic glycolysis (Renkin, 1984). Further, it has been suggested by some investigators that normally there are tissue areas on the borderline of hypoxia, producing vasodilator metabolites in sufficient quantities to maintain adequate tissue oxygenation overall. In the contracting myocardium a few tissue areas with $PO_2 < 5$ mmHg were found using an oxygen microelectrode (Schubert *et al.*, 1978). Whether such areas were below critical PO_2 was not determined. As noted above, tissue PO_2 in resting skeletal muscle is well above the critical level and when blood is completely stopped, 10–45 seconds elapse before a shift to anaerobic glycolysis begins (Toth *et al.*, 1996; Richmond *et al.*, 1999). The circumstances under which a shift to anaerobic metabolism serves to regulate blood flow appear to be limited but, as noted above, this mechanism appears to prolong reactive hyperemia when the intra-organ oxygen stores are exhausted. It could also be involved in the vasodilation with hypoxia if the critical PO_2 for the tissue is reached. Whether it is important in functional hyperemia is less clear. A recent study in the intact pig heart using a non-invasive optical technique showed that increased workloads do not cause significant myoglobin desaturation, and thus oxidative metabolism is maintained. Earlier studies with more invasive methods suggested that an anaerobic shift might occur (Arai *et al.*, 1999). In respect to skeletal muscle, there is evidence that anaerobic metabolism and lactate production increase in exercise. However, it has been suggested that the

increase is not due to a drop in PO_2 below critical levels (Richardson *et al.*, 1998). Another group has reported that anaerobic ATP production increases before maximal exercise is reached, but this is not always accompanied by an increase in H^+ concentration due to an increased rate of removal (Roussel *et al.*, 2003). Based on these observations, it is not clear at present that anaerobic metabolism contributes importantly to functional hyperemia in the myocardium or skeletal muscle.

OXYGEN-SENSITIVE MECHANISMS

In recent years much new information has become available on intravascular mechanisms that respond directly to changes in oxygen tension or blood oxygen content rather than indirectly through changes in parenchymal cell metabolism. A response of the microvasculature to PO_2 changes in the immediate environment has been shown in a variety of experiments. Increasing the PO_2 in a suffusate bathing a surgically exposed microvascular bed causes constriction of the arterioles (Duling, 1972; Prewitt and Johnson, 1976). The role of prostaglandins in mediating the microvascular response to oxygen has been highlighted by studies showing that lowering PO_2 levels increases the release of prostaglandins from endothelial cells (Busse *et al.*, 1984) and blockade of prostanoid production decreases the vasodilation during systemic hypoxia as shown in Figure 9.4. Also, studies on isolated arterioles from rat cremaster muscle revealed that elevating oxygen above normal levels causes vasoconstriction due to inhibition of release of prostaglandins from the endothelium (Messina *et al.*, 1994). The effect is found over a wide range of PO_2 values, from 15 to 660 mmHg, which would provide a means by which elevation of oxygen above normal levels exerts a vasoconstrictor effect on the arterioles. Nitric oxide has also been implicated as a mediator of the oxygen dependency of vascular tone by a wide variety of studies showing that it is released from endothelial cells at low oxygen tension (Pohl and Busse, 1989), and that the vasodilation of resistance vessels to lowered oxygen in certain vascular beds is abolished by blockade of nitric oxide synthase (Blitzer *et al.*, 1996). It has also been shown that a P-450 metabolite of arachidonic acid, 20-HETE, is involved in the vasoconstrictor response to elevated oxygen tension (Roman, 2002).

Evidence that endothelial-derived adenosine is in part responsible for the dilation of rat femoral vessels during systemic hypoxia has been described above (Figure 9.4). Recently, evidence has been developed that the red cell itself may participate in the vascular response to lowered oxygen. It has been shown that hemoglobin deoxygenation in the passage of blood through the microcirculation causes release of ATP from the red blood cell (Sprague *et al.*, 1996; Ellsworth, 2004), which may act on the arteriole. ATP could also act on the venular endothelium to cause release of prostaglandins, which could in turn diffuse to adjacent arterioles as reported by Hester and Hammer (2002), providing a feedback mechanism linked to HbO_2 saturation in the microcirculation. The vascular network also possesses the capability of transmitting hyper- or depolarization, and it has been suggested that a vasodilator response is conducted upstream from the capillary network to the arterioles in contracting muscle (Berg *et al.*, 1997). During extreme hypoxia an additional mechanism comes into play. When PO_2 of the vascular smooth muscle falls below the critical level for oxidative metabolism, cytochrome a,a3 in the smooth muscle mitochondria may function as a sensor to cause relaxation of the vessel (Katayama *et al.*, 1994).

MECHANISMS OF FUNCTIONAL HYPEREMIA

In the absence of an anaerobic shift, certain mechanisms of vasodilation described above may contribute to functional hyperemia – for example, those related to deoxygenation of the red cell. Those related to a drop in PO_2 of the arterial vessels may not contribute greatly unless increased flow does not offset the increased PO_2 radial gradient. Lash and Bohlen (1987) showed that periarteriolar levels were only transiently reduced during muscle contraction. The fraction of functional hyperemia in muscle that can be attributed to a drop in PO_2 is not clear. When a resting muscle of the dog hind limb was perfused with blood having the same PO_2 as that of exercising muscle, the fall in vascular resistance was about one-third that seen during contraction (Ross *et al.*, 1964). An important difference, however, is that the intraluminal PO_2 in the arterial vessels would be lower with

perfusion of the muscle with hypoxic blood as compared to muscle contraction. Considerable effort has also been devoted to identifying the vasodilator metabolites responsible for functional hyperemia. Adenosine, being a breakdown product of ATP, has been a major focus, especially in the myocardium, but the results with blocking agents and estimates of changes in interstitial fluid leave questions regarding its importance in the absence of a shift to anaerobic metabolism (Tune *et al.*, 2000).

It has been recognized for many years that increased oxidative metabolism will elevate the levels of interstitial CO_2, H^+ and inorganic phosphate, all of which have some vasodilator capacity, as does K^+ released from the muscle fibers with depolarization of the muscle fibers. The resultant increased osmolarity of the interstitial fluid also has a vasodilator effect (Sparks, 1978; Johnson, 1986). More recently nitric oxide, prostanoids, endothelium-derived hyperpolarizing factor and ATP released from the red cell with hemoglobin desaturation and mechanical deformation have been proposed as mediators of exercise hyperemia (Clifford and Hellsten, 2004; Ellsworth, 2004). While the multiplicity of candidates may be a reason for skepticism, it can be argued that the importance to the organism of maintaining an adequate oxygen delivery with increased demand would justify development of redundancy in flow regulation.

MECHANICAL STIMULI FOR FLOW REGULATION

As noted above, when arterial pressure to the myocardium, skeletal muscle and other vascular beds decreases, there is a compensatory autoregulatory dilation of the resistance vessels that returns blood flow toward its previous level. Although this dilation tends to maintain constant oxygen delivery to the tissue, it depends importantly on the myogenic response, which is in turn dependent on the intravascular pressure in the resistance vessels. This mechanism appears to react in such a way as to maintain a constant circumferential wall tension or hoop stress in the resistance vessels (Johnson, 1980). While stretch of the entire cell would not provide a feedback signal that allows the vessel to become narrower with increased pressure, a sensor element in series with the contractile machinery of the cell would fill such a role. Recent studies reviewed by Hill *et al.* (2001) have demonstrated that receptors at the cell surface, such as integrins, provide a mechanism for an increase in wall tension to be communicated from the extracellular matrix to the smooth muscle cell. There is evidence that mechanically sensitive ion channels, voltage operated Ca^{2+} channels and probably other mechanisms as well are involved in transduction of the mechanical force to the increased contractile activity seen in the myogenic response.

Another mechanism responsive to mechanical stimuli, and better known, causes dilation of the arterial vessels when flow is increased. This is due to release of EDRF (endothelial-derived relaxing factor) when wall shear stress on the luminal surface of the endothelium rises. It serves to augment flow in collateral vessels when a large supply vessel is occluded, and is responsible for the dilation in the arterial vessels outside the muscle itself during muscle contraction (Smiesko and Johnson, 1993). Flow-induced dilation is due to prostanoids in rat cremaster muscle (Koller *et al.*, 1989), but in other vascular beds (such as the spinotrapezius muscle) nitric oxide appears to be mainly responsible (Friebel *et al.*, 1995).

INTEGRATIVE ASPECTS OF FLOW REGULATION

From the foregoing description of local regulation of blood flow, which admittedly is far from exhaustive, it is evident that a wide variety of regulatory mechanisms responding to a number of different stimuli are involved in determining the flow of blood to the tissues. The individual mechanisms of flow regulation operate in a manner that is sometimes synergistic and other times antagonistic. Present evidence suggests that a wide variety of mediators act in concert during functional hyperemia, providing a high degree of redundancy for this important function. Adenosine and prostanoids together are responsible for most of the dilation in the femoral resistance vessels in hypoxia (Ray *et al.*, 2002). In another intimate relationship, 20-HETE enhances the myogenic response in the kidney and other vascular beds (Roman, 2002). Conversely, mechanisms can be put into opposition

as when venous pressure is elevated, reducing blood flow which would cause a reduction in tissue and microcirculatory PO_2 to cause vasodilation while at the same time increasing intravascular pressure in the terminal arterioles to cause vasoconstriction through the myogenic response (Johnson, 1980). Studies of the interactions among regulatory mechanisms have utilized data on the responses of isolated vessels to specific stimuli. Models have been developed in which the combined effects of myogenic, metabolic and wall shear stress-activated mechanisms to a specific perturbation can be predicted (Liao and Kuo, 1997; Cornelissen *et al.*, 2002). Information on the gain of these mechanisms at different levels of the vascular bed has suggested, for example, that a balance is required between shear stress-induced and metabolic mechanisms for effective myogenic regulation of blood flow with alteration of arterial pressure.

While local flow regulatory mechanisms are very important determinants of blood flow to individual organs, the central nervous system also exerts an important influence through the sympathetic nerve supply. In most organs, when the sympathetic nerves are severed the blood flow increases significantly but may subsequently return essentially to the previous level. The interaction of central and local mechanism is in some instances synergistic and in other cases antagonistic. In resting skeletal muscle, the myogenic mechanism is weak but can be enhanced by stimulation of the sympathetic nerve supply; as a consequence autoregulation of blood flow with alteration of arterial pressure becomes more pronounced and flow may in some instances significantly increase when arterial pressure is reduced (Ping and Johnson, 1994). At times, local and central mechanisms may oppose each other. When sympathetic nerves to muscle or intestine are stimulated, flow decreases and then returns partially toward the control level. The partial recovery has been shown to be due to the fall in oxygen level in the organ, since it can be prevented if local oxygen levels in the microcirculation and tissue are maintained with elevation of the suffusate oxygen (as shown by Boegehold and Johnson, 1988). The physiological utility of this antagonism is most evident in exercising muscle. With the onset of exercise there is a generalized increase in sympathetic nerve activity, which reduces blood flow to inactive muscle while having little effect on the hyperemia of the contracting muscle – a condition termed functional sympatholysis.

These examples serve to illustrate the complexity of what we term 'flow regulation' in the peripheral circulation. It is now well recognized that not all the regulatory mechanisms are specifically directed towards control of blood flow. It is also apparent that none of the individual mechanisms can act in isolation. The impact of a specific mechanism may be affected by other mechanisms, either directly or indirectly, through a change in wall shear stress, intravascular pressure, volume flow or oxygen levels in the blood stream or tissue. As we learn more about the regulatory mechanisms, we cannot help but be struck by the manner in which these mechanisms complement each other and are closely attuned to the specific physical and biochemical characteristics of the peripheral vasculature and the particulate suspension that flows through it.

How can information on local regulation aid in the development of blood substitutes? First, it is evident that physical or biochemical properties of the substitute that differ significantly from whole blood could trigger responses of a number of regulatory mechanisms, some of which may not be directed toward delivery of oxygen to the tissues or could actually oppose it. Based on this assumption, it seems likely that there would be fewer potential problems with a substitute that mimics as closely as possible the physical and biochemical characteristics of whole blood. A second approach would be to define as precisely as possible the characteristics of a proposed blood substitute that differ qualitatively or quantitatively from whole blood, and use that information to evaluate the effect on known regulatory mechanisms. Incorporating those characteristics into models such as those described above for the coronary circulation could be a useful starting point. This could be coupled with experimental studies in which the effect of each of the unique characteristics of the substitute on vascular function is studied and verified separately. As described elsewhere in this volume, certain aspects of what is being suggested here are already being incorporated into the development of blood substitutes in a number of laboratories. It is hoped that this review of local regulatory mechanisms will encourage further efforts in this direction.

EDITOR'S SUMMARY

The mechanisms of delivery of oxygen from the atmosphere to sites of metabolism (mitochondria) are the result of millions of years of evolution. Decades of research are now bringing into focus a unified description of the multiple interacting physiological and biochemical mechanisms that result in a highly redundant system of oxygen transport. As this chapter points out, a blood substitute whose properties are different from those of blood is likely to trigger responses that might appear unexpected or contradictory. In the past only the affinity of hemoglobin for oxygen has been considered to be important in this regard, but clearly this is only one of many key features of the system. It is evident that a completely satisfactory 'blood substitute' cannot be designed until enough of the normal system of regulation of oxygen transport to tissues is understood to prevent adverse physiological mechanisms from being engaged.

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SECTION

3

Clinical Applications

Since no blood substitute products are currently approved for clinical use in the US or Europe, the anticipated clinical applications are limited to those for which regulatory approval is sought. However, it is certain that when products are readily available to clinicians and researchers, new applications, not currently anticipated, will emerge. This section begins with a discussion of crystalloid use, which is the most commonly used volume expander today. It then delves into applications where large amounts of blood are used, including trauma and surgery. Finally, it considers hemodilution and the treatment of ischemia – applications in which blood is not currently used, but for which cell-free oxygen carriers might provide clinical benefit that no current product is able to provide.

Chapter 10

Clinical Indications for Blood Substitutes and Optimal Properties

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INTRODUCTION

The clinical indications for which red cell substitutes would be used are still not defined because no product is approved; the nature and properties of the products that will eventually be approved are still therefore speculative, as are the clinical indications for which they might be beneficial. As work on blood substitutes has progressed over the past two decades, the safety of allogeneic blood transfusions has improved dramatically (see Chapter 2), and the requirements for regulatory approval have sharpened (see Chapter 3). Since the risk of transfusion is now quite low, the burden of proof will be on the developer of any new agent to show that it is as safe and efficacious as current red cell transfusion.

In spite of the changing landscape of blood substitute development and the lack of availability of FDA-approved products, it is nevertheless useful to consider the clinical applications for which these solutions might be useful. This exercise is useful in planning specific formulations. This chapter will consider a few of the possible applications for hemoglobin-based red cell substitutes (Table 10.1).

Table 10.1 Potential clinical applications for blood substitutes

Trauma/resuscitation
Elective surgery – hemodilution or blood replacement
Red cell incompatibility
Ischemic disease and angioplasty
Extracorporeal organ perfusion
Cell culture media
Hematopoietic stimulation
Cardioplegia
Sickle-cell anemia
Tumor therapy
Chronic anemia
Research

TRAUMA/RESUSCITATION

One of the most obvious clinical applications for hemoglobin-based red cell substitutes is in emergencies such as trauma. Among the factors that determine the long-term survival after resuscitation from hypovolemic shock is the duration of the shock state (Baker *et al.*, 1985). Messmer and his colleagues (Messmer, 1988) have conducted extensive studies of the microcirculation in shock, and have pointed out that under the influence of the sympathetic nervous system organ flow is redirected to protect vital organs.

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Traumatized tissue can be excluded from this control, however, and blood actually may be directed preferentially to sites of bleeding, with consequent hematoma formation. This redistribution of blood flow may protect systemic pressure so that in some instances the degree of shock may not be clinically apparent. Thus, the survival after hemorrhage from severe trauma declines dramatically after the first hour. This critical period has become known as the 'golden hour' (Belzberg, 1989; unpublished comments). Experience during the Korean and Vietnamese conflicts has convinced military physicians that this period is also critical on the battlefield (Mosebar, 1989).

The objective of posthemorrhage fluid therapy is to replace lost volume and electrolytes and to re-establish the flow of oxygen to ischemic tissue. This requires restoration of cardiac output as well as tissue reperfusion. Treatment with both colloid and crystalloid (electrolytes) is needed. Available colloids include human serum albumin, dextran, and hydroxyethyl starch. Albumin is not used routinely because of its high cost and short supply. Dextran solutions have antithrombotic properties, but their half-life in the circulation is from 2 to 6 hours, and they carry the risk of anaphylaxis.

Recent studies in the microcirculation (Kerger *et al.*, 1996, 1997; see also Chapter 7) have shown that a critical parameter in the hamster is the functional capillary density (FCD), a measure of local tissue perfusion. Maintenance or re-establishment of the FCD may be more critical than restoration of the mean arterial pressure, the usual index of successful resuscitation. It appears that restoration of normal aerobic metabolism in the shortest possible time after the onset of shock is of the highest priority, and the optimal resuscitation solutions would be those that have this effect.

There can be little doubt that the ideal emergency resuscitation fluid would be one that carries oxygen in addition to providing blood volume expansion. Whole blood or red cells are not routinely used as a first-line resuscitation fluid because of the requirement to cross-match, delaying their availability by an hour or more. Typically, trauma patients receive large volumes of crystalloid (saline or Ringer's lactate) prior to blood. Thus, a blood substitute could be aimed at those trauma victims with hypovolemic shock who might otherwise die without immediate restoration of their oxygen supply. Of course, the determination of which patients fall into this category is still problematic.

Since selection of patients for specific parenteral fluid treatment is controversial, it is premature to speculate on which would be candidates to receive blood substitutes if they were available. Trauma specialists have disagreed for years on whether crystalloid or colloid is preferred in blood-loss shock (Messmer, 1988), but it seems that most patients are eventually given electrolytes, colloids, blood, red cells, and clotting factors at some time during their therapy.

In routine clinical practice, Ringer's lactate or saline are the most widely used primary volume expanders. In order to restore the cardiac output with crystalloid, three to four times the volume of lost blood must be given (see Chapter 12). Some of this hypo-oncotic material is lost into the interstitial space, and tissue edema can result. The considerable amounts of oncotically active molecules lost in blood-loss shock must be replaced eventually. Prospective, randomized studies have shown that in fact hemodynamics are restored faster, with less tissue edema, when dextran 70 is the first fluid used instead of Ringer's acetate (Modig, 1983, 1986). Table 10. 2 summarizes some differences between crystalloids and colloids,

Table 10.2 Comparison of some characteristics of crystalloids, colloids and HBOCs

	Crystalloid	Colloid	HBOC
Intravascular volume effect	Poor	Better	Better if increased COP
Restoration of FCD	Poor	Better	Best
Interstitial volume effect	Better		Better if decreased COP
Pulmonary edema	Equal	Equal	?
Peripheral edema	Common	Rare	?
Immune reactions	Uncommon	Occasional	Depends on formulation
Cost	Inexpensive	Expensive (albumin) Moderate (starch)	Probably high

and considers how hemoglobin-based oxygen carriers (HBOCs) might compare with those two traditional classes of plasma expanders.

Another solution intended for use in the 'golden hour' is hypertonic saline, sometimes combined with dextran 70. These solutions may contain up to 7.5 g/dl of sodium chloride. A number of investigators have shown that when small volumes of such solutions are given to animals after the induction of hypovolemic shock, the cardiac output is restored almost immediately (Smith *et al.*, 1985; Kramer *et al.*, 1986; Maningas *et al.*, 1986; Kreimeier and Messmer, 1987). In addition to the volume expansion properties, lower molecular weight dextran 40 also has antithrombotic properties that make it attractive in vascular obstructive disease, hyperviscosity syndromes, and cerebral vascular insufficiency (Messmer, 1988). The Institute of Medicine of the Academy of Science reviewed the practices of shock therapy, concluding that hypertonic/hyperoncotic solutions should be the first choice of therapy of acute blood-loss shock (Pope *et al.*, 1999).

Hemoglobin solutions have the potential to combine the advantages of crystalloids and colloids. It would seem that most of the desired properties of hypertonic saline-dextran, albumin, and whole blood could be combined in a single solution of hemoglobin and electrolytes. However, specification of the properties of this 'ideal' solution might be controversial.

The use of type O ('universal') donor cells became widespread during World War II (Camp and Dawson, 1974). In the Vietnam conflict, between 1967 and 1970, 100 419 transfusions were given, with only nine reported deaths caused by mismatched blood resulting from clerical error (Camp and Dawson, 1974; Camp, 1975). By themselves, these figures would not seem to support the development of blood substitutes on the grounds that cross-matching is not necessary. However, if a hemoglobin-based red cell substitute is as useful in the battlefield setting as uncross-matched blood or type O cells, then its use could have a tremendous impact on the provision of transfusion services by the military.

Recent military experience in Grenada, Panama and the Middle East has suggested that the military requirements for blood could be changing. That is, conflicts now tend to be of high intensity but short duration, placing a potential stress on the blood supply. Furthermore, conflicts tend not to be concentrated in any particular area, such as the front lines of World War II, and therefore casualties need to be evacuated, sometimes over long distances, to arrive at a medical care facility. Thus the importance of the 'golden hour' in the military setting. Hemoglobin-based red cell substitutes could be stockpiled for long periods, presumably, and could be more readily available than whole blood, which requires an elaborate collection, processing and distribution system.

In the civilian setting, the use of uncross-matched blood or type O cells is much less frequent. In one major center (Yale University) in the years 1974–1976, 121 patients received type O cells without further cross-matching (Blumberg and Bove, 1978). No untoward effects as a result of the transfusions were noted, and the procedure seemed to have no effect on the overall clinical outcomes. The authors of this study concluded that many of the patients could have been managed with conventional volume expanders and could have waited for proper cross-matching for transfusion. They also pointed out that when uncross-matched blood or type O red cells are available, they are used. Clearly, if red cell substitutes devoid of the risks of incompatibility and disease transmission were available, they would be used also.

ELECTIVE SURGERY

In the United States, it is during the perioperative period that most blood and red cells are used. Indications for transfusion have included a hemoglobin concentration less than 10 g/dl (Mollison and Engelfriet, 1997), but this rule is being re-evaluated at present in view of the diminishing blood supply and the recognition of the complications of transfusion (Consensus Conference, 1988). However, surgical studies suggest that the postoperative period can be shortened and, perhaps, made safer if hemoglobin concentrations are not allowed to fall to less than 10 g/dl. Although animal studies suggest that the hemoglobin concentration could be reduced safely to about 25 per cent of its normal value (Wright, 1975), clinical experience suggests that the hemoglobin concentration should not be allowed to drop below 8 g/dl (Carson *et al.*, 1988). In addition to the prevention of anemia, the outcome of some surgical procedures is improved if blood viscosity can be reduced (Mandel, 1986). In these procedures, the combination of low

viscosity and maintained oxygen-carrying capacity might optimize the transport of oxygen to tissue.

The use of an oxygen-carrying red cell substitute could be invaluable in managing elective surgical procedures. Blood could be collected at the start of the operation and replaced by the oxygen carrier. The removed autologous blood could then be used as needed, either during or after surgery.

RED CELL INCOMPATIBILITY

Certain patients develop antibodies to many red cell surface antigens either because they have received multiple transfusions, sometimes over an entire lifetime, or because the existence of unusual antigens has led to minor incompatibilities in the past, with resultant elaboration of antibodies. Some patients simply cannot receive red cell transfusions without undue risk of a fatal reaction. Perhaps blood substitutes could play a role in some of these instances. However, it must be kept in mind that the products being developed now are only oxygen-carrying solutions; they cannot, at present, substitute for other important functions of blood – namely, hemostasis and immunity.

ISCHEMIC DISEASE AND ANGIOPLASTY

Tissues that are poorly perfused with blood and therefore receive a marginal supply of oxygen may benefit from increased perfusion with cell-free oxygen carriers. Examples might include ischemic ulcers seen in diabetic or sickle-cell anemia patients, and cerebral ischemia. One report (Iwai *et al.*, 1989) demonstrated improved healing of ischemic ulcers when they were bathed in oxygenated perfluorodecalin.

Morbidity during and after percutaneous transluminal coronary angioplasty (PTCA) may be reduced if the myocardium can be perfused with an oxygen carrier. Perfusion with blood is limited because of blood's high viscosity, but perfluorocarbon emulsions were licensed for this application. Solutions of modified hemoglobin with even lower viscosity have been used experimentally (Rossen *et al.*, 1987). Although this appeared to be a very important application for blood substitutes, since a large (and increasing) number of coronary angioplasties is performed in the United States each year, the product was discontinued by its manufacturer shortly after its introduction because it was not well-received by physicians. This 'first generation' product had to be mixed at the bedside prior to infusion, and efficacy was felt to be marginal (Gould *et al.*, 1986).

EXTRACORPOREAL ORGAN PERFUSION

The preservation of organs for transplantation is an increasing requirement as graft success improves. Hearts, livers and kidneys might survive better if they could be perfused with an oxygen-carrying solution. Recent work with isolated perfused rabbit hearts has indicated that much more oxygen can be delivered to the myocardium with a hemoglobin-based blood substitute compared with Krebs–Henseleit solution, even if the latter is equilibrated with 100% oxygen (MacDonald and Winslow, 1992).

Hemoglobin solutions may be useful in preserving severed limbs for reattachment. Bonhard (1988) discussed the use of pyridoxylated hemoglobin in this regard, and suggested that post-ischemia syndrome can be prevented. Steinau and Elert (1980) showed that adenosine triphosphate (ATP) levels were higher and lactate concentrations lower in limbs perfused with hemoglobin solutions rather than with Ringer's lactate.

CELL CULTURE MEDIA

The recent explosion in cell-cloning technology has dramatically increased the use of cultured mammalian cells for commercial as well as therapeutic and research purposes. It is possible that the quality of these cells and their yield of recombinant gene products can be increased by the use of oxygen-carrying culture media.

HEMATOPOIETIC STIMULATION

Hawkins and Johnson (1939), Amberson *et al.* (1949) and Gould and colleagues (Moss *et al.*, 1988) all noted stimulation of erythropoiesis after administration of cell-free hemoglobin. It is likely that this is a result of the ready availability of heme iron when administered as hemoglobin.

Recombinant human erythropoietin also stimulates red cell production in the preoperative period. Goodnough *et al*. (1989) showed that 41 per cent more autologous blood could be collected from surgical candidates when erythropoietin was administered. Their patients were all given oral iron sulfate during the 21 days before surgery. The combined use of erythropoietin and cell-free hemoglobin solution could provide a potent stimulus to endogenous red cell production.

CARDIOPLEGIA

During certain surgical procedures, such as cardiac valve replacement, repair of congenital anomalies, or aneurysm resection, it is necessary to slow or stop the normal heart action. This is done by perfusing the myocardium with various electrolyte solutions and lowering the temperature. Obviously, solutions that transport oxygen to tissues would be desirable since they could both prolong the time allowed for the surgeons to operate and also speed recovery of function after the procedure.

Elert and Otterman (1979) perfused dog hearts with a solution of pyridoxylated hemoglobin and electrolytes. They induced cardiac arrest with this solution, and measured ATP and phosphocreatine. They were able to show a 20-minute gain in the length of time for which hearts could be stopped, and they had no difficulty in restarting them. On histologic examination, they found less endothelial swelling than in those hearts perfused without hemoglobin.

SICKLE-CELL ANEMIA

Therapy of sickle-cell anemia has a long and controversial history. In this chronic hemolytic anemia, patients 'adapt' to a low hemoglobin concentration, and anemia *per se* is not the main problem. Rather, the rheologic consequences of deformed red blood cells cause morbidity and mortality. The degree of pathologic damage in sickle-cell anemia, moreover, is highly variable from patient to patient, and the clinical course in an individual is completely unpredictable. Some patients may be almost asymptomatic throughout their lives, while others can be severely debilitated. This unpredictability makes the design of clinical trials difficult.

Periodically, sickle-cell patients experience painful 'crises' – episodes characterized by intense pain that can be generalized or localized to specific organs or regions of the body. The pathologic events that underlie these crises involve obstruction of the microvasculature by deoxygenated sickled red blood cells. This obstruction prevents adequate oxygenation of the tissues served by the obstructed capillary beds, and the pain is therefore ischemic.

The red blood cells of patients with sickle-cell anemia deform ('sickle') when they are deoxygenated, when the pH drops or when they are dehydrated. When any of these condition occurs, the viscosity rises dramatically and circulation slows. Thus, at a given hematocrit, sickle-cell blood has a higher viscosity than normal blood (Charache and Conley, 1964). Oxygen transport can be improved in sickle-cell anemia patients by exchange transfusion with normal red cells (Miller *et al*., 1980), because better microvascular blood flow can be achieved without reducing the hemoglobin concentration.

Dilution with a hemoglobin solution may be even more effective than exchange transfusion with whole blood. The idea is especially attractive, since one of the contraindications to exchange with normal blood is the membrane antigen sensitization that eventually occurs after multiple transfusions. In addition, cell-free hemoglobin might be effective in perfusing obstructed capillary beds. *In vitro* observations suggest that a hemoglobin–polyoxyethylene conjugate may reverse capillary occlusion with sickled cells (Yabuki *et al*., 1988). Limited clinical trials using modified hemoglobins to treat sickle-cell crises have been encouraging (Feola *et al*., 1992; Gonzalez *et al*., 1997).

One difficulty with this application in the United States and Europe is that the sickle-cell patients who are in most need of therapy are those with the poorest venous access, and thus hemodilution with cell-free solutions might still be difficult. An additional factor that potentially limits this application is that although each individual sickle-cell patient is very dependent on the health-care system, the total number of patients may not justify the development of commercial products. In African countries, however, where the incidence of sickle-cell anemia is much higher, these products could have a major impact on health care. In these countries, the risk of transmission of HIV by blood transfusion is extremely high.

TUMOR THERAPY

Some treatments of soft tissue tumors involve local destruction of malignant tissue by activated oxygen. Thus, any method for increasing tissue oxygen tension could be viewed as an adjunct. The perfluorocarbons have been suggested for this application but, if proven safe, hemoglobin could also be useful for some types of therapy. In this case, the optimal product would have the highest *P*50 possible since the solution would be oxygenated outside of the body and infused directly into the circulation of the tumor.

An alternative approach has already been shown to be effective in animals. Drugs (clofibrate, bezafibrate and gemfibrozil) that reduce oxygen affinity dramatically (rise of 10–20 Torr in the *P*50 value) were administered to tumor-bearing mice. Marked sensitization to irradiation was observed when these animals were given supplemental oxygen to breathe (Hirst *et al*., 1987).

If a high 'arterial' oxygen tension is achievable, then perfluorocarbons might be the perfusion medium of choice since photoactivation of heme-containing drugs also could be used (hemoglobin would absorb much of the delivered light energy). These applications could be fairly widespread, eventually, and commercialization might be attractive, since the volume of agent would be much less than in high-volume resuscitation or hemodilution applications.

CHRONIC ANEMIA

Chronic anemia would not seem to be a good indication for blood substitutes. First, patients usually adapt to chronic anemia just as high-altitude natives seem to become acclimatized to chronic hypoxia. The inability of the currently developed blood substitutes to provide coagulation factors, platelets or leukocytes would not seem to be a major drawback, especially in patients with chronic hemolysis, red cell aplasia, cancer, or other types of chronic disease.

If a red cell substitute were developed for chronic anemia, it would need to have the longest possible plasma retention to eliminate the need for excessively frequent infusions. The products that might be candidates to fill this need would be encapsulated or conjugated hemoglobin or derivatives with a very high molecular weight. Most efforts have been directed toward development of perfluorocarbons with short retention times, and of hemoglobin solutions with intermediate times.

RESEARCH

Thirty years ago, Geyer (1975) reviewed the potential uses of blood substitutes. The list of anticipated clinical applications was much like the one above, and was the driving force behind commercialization and therefore the availability of products. However, Geyer also listed prominently a number of research areas that will benefit from the availability of these products. They will be extremely useful in basic studies of hematopoiesis, protein metabolism, all aspects of oxygen transport, including the microcirculation, and utilization of oxygen by tissues, and particularly the differences between specific organs. This research will not only advance basic knowledge of biochemistry and physiology but will also doubtless give rise to new clinical uses that are not appreciated now.

REQUIREMENTS FOR A BLOOD SUBSTITUTE

Some of the required properties of red cell substitutes are obvious. They must be sterile, free of endotoxin and viruses, non-toxic and efficacious, and it must be possible to store them for reasonable periods. The methods used to produce them must be tractable for industrial scale-up, and not too expensive. Lot-to-lot variation and immunogenicity must be minimal. These requirements can pose serious industrial problems, particularly when large batches must be prepared in a routine fashion. Some of the other properties would be tailored to the individual products under development, and their specifications are more controversial.

It is clear that the properties of a hemoglobin solution intended for parenteral use should be matched to the clinical indication. For example, administration in a case of chronic, refractory anemia would require a long plasma retention and normal colloid osmotic pressure. A solution for resuscitation from acute blood loss should have an increased colloid osmotic pressure but may have short plasma retention because blood would be available to the patient within several hours of injury in most cases. The patient who is

unable to maintain a normal alveolar oxygen tension might do better with a reduced *P*50. In some applications (such as organ preservation for transplantation) the properties are probably less critical, because greater control of oxygenation and temperature, for example, is possible.

Oxygen capacity

A red cell substitute must, of course, carry oxygen, but it is difficult to specify how much. Perhaps the oxygen-carrying capacity of blood with a normal hemoglobin concentration is not necessary, but there are no guidelines as to the minimal hemoglobin concentration required for health. For years surgeons and anesthesiologists have used the '10-gram rule' in the belief that a hemoglobin concentration of less than 10 g/dl will impair tissue healing. As pointed out earlier, perhaps this figure can be safely dropped to 7–8 g/dl.

Recent work, reviewed later, has suggested that when the oxygen carrier is in the plasma space, oxygen capacity may need to be limited to prevent engaging local autoregulatory, vasoconstrictive mechanisms (Intaglietta *et al*., 1996). This raises a further question: how will the clinician re-evaluate the 'transfusion trigger', given that cell-free oxygen carriers transport oxygen in ways fundamentally different from those of native red blood cells?

Oxygen affinity

Another difficult specification is the oxygen affinity of the prospective red cell substitute. As a first approximation, it would seem logical that the *P*50 should be that of normal human blood, but it is known that even in natives of high altitude, variation around the normal *P*50 of about 28 mmHg does not seem to affect oxygen transport (Winslow and Monge, 1987).

Although the determination of the lowest and highest values for *P*50 for blood is approximate at best, some rough limits can be set. Abnormal human hemoglobins with *P*50 values in the area of 19 mmHg lead to polycythemia, showing that oxygen delivery to the renal site of erythropoietin production in these cases is compromised. The upper boundary for the acceptable *P*50 is much more controversial. Some workers believe that there is no limit to the benefit of increased *P*50, but this depends on the ability of the lung to oxygenate blood. That is, if 100 per cent saturation is reached in the lung, it is likely that the higher the *P*50, the more easily oxygen can be unloaded in the tissues. This would be the case in a patient undergoing elective surgery in which arterial oxygen tension is controlled. However, if pulmonary oxygenation is compromised in any way, the uptake in the lung becomes an issue and a lower *P*50, rather than higher, is beneficial (Winslow *et al*., 1984). A high *P*50 would also be desired in tumor therapy to maximize tissue oxygen tension.

Less obvious than these considerations for blood is the optimal *P*50 for a cell-free oxygen carrier. Theoretical approaches to the question predict that lower, not higher, *P*50 would be advantageous in limiting diffusive delivery of oxygen to cell walls (Winslow and Vandegriff, 1997). Studies in the hamster microcirculation have now confirmed this theory (Tsai *et al*., 2003). Less obvious than these considerations is the importance of the Bohr effect, cooperativity and carbon dioxide transport. It should be noted that only one relevant study of carbon dioxide transport in a potential red cell substitute has been reported (Vandegriff *et al*., 1991).

Viscosity

The viscosity of a blood substitute is a critical issue, since the bulk viscosity of whole blood is an exponential function of hematocrit. Some workers have reported that oxygen delivery to the brain may be compromised when the hematocrit is elevated even to the high-normal range (Goodnough *et al*., 1993). At equivalent hemoglobin concentrations, crosslinked hemoglobin has a lower viscosity than whole blood at the low shear rates found in capillaries. When the blood substitute is polymerized hemoglobin, the viscosity increases with the degree of polymerization. Thus, there is some theoretical basis for the notion that the viscosity of a red cell substitute should not be higher than that of blood, particularly in the presence of red blood cells in proportions likely to be found in clinical practice.

Red cell suspensions are non-Newtonian in their flow patterns; *in vivo* flow is turbulent, and red cells stream. These two properties are dependent on the diameter of the vessel. Turbulence is

probably important to red cell oxygen transport because of stirring in the area immediately surrounding the cell. In contrast, cell-free hemoglobin solutions will be homogeneous with respect to flow. The effect on oxygen delivery in tissue capillary beds is unpredictable.

Plasma retention

A red cell substitute must have a reasonable intravascular persistence. Although the length of time the oxygen carrier should remain in the circulation is not defined clearly (Greenburg *et al.*, 1982), a period of 20–40 hours is often cited. Vagueness in this specification is not surprising, since the clinical indications for such solutions have not been determined. Additional considerations are the toxicity and route of removal from the circulation. Modified hemoglobins are not removed primarily by renal mechanisms (Keipert *et al.*, 1989); rather, these materials are taken up by the macrophage-monocyte (reticuloendothelial) system and other tissues. If the solutions are toxic, then the least possible exposure would seem desirable and they should be used only in emergency situations – probably for resuscitation. If the solutions are not toxic, then a longer intravascular persistence would seem permissible, and a wider clinical application might be established.

The polymerized hemoglobin products and hemoglobin conjugated to dextran, PEG or other polymers have the longest intravascular retention times. Hemoglobins crosslinked at intramolecular sites all seem to have retention times three to five times longer than unmodified hemoglobin, but the actual times are species- and dose-dependent (Hess *et al.*, 1989). The retention in humans will not be known until appropriate clinical trials are reported, and doses can be administered which are higher than those used in the limited safety trials carried out to date.

Colloid osmotic pressure

It is important to emphasize the fundamental differences between solutions that exert a colloid osmotic pressure (colloids) and those that exert only an osmotic pressure (crystalloids). Colloid solutions are those that contain particles that are osmotically active but which do not diffuse across biologic membranes. Crystalloids are also osmotically active, but the molecules can diffuse across membranes. Thus, both solutions expand the intravascular space immediately, but the effect of colloid solutions persists longer.

One of the chief arguments for the polymerization of hemoglobin has been that when the molecular weight is increased the concentration of molecules is less, even though the oxygen carried is the same. Thus, pyridoxylated, polymerized hemoglobin can be infused into animals to achieve a final concentration of about 10 g/dl and a normal colloid osmotic pressure. In this case, there are no major shifts of water between the interstitial and intravascular spaces. In contrast, unpolymerized hemoglobin with a molecular mass of 64 000 Daltons has increased colloid osmotic pressure; only about 7 g/dl can be achieved because infusion requires a shift of water from the interstitium into the intravascular space to maintain oncotic equilibrium. Some workers in the trauma field believe hyperoncotic solutions are preferred for resuscitation because volume expansion can be achieved more rapidly than with the infusion of large quantities of isotonic solutions. Menu *et al.* (1985) actually showed improved survival in rats with cell-free hemoglobin when the concentration was 12.5 g/dl rather than 7.0 g/dl, despite (or maybe because of) a very high colloid osmotic pressure.

Storage stability

Obviously, storage stability is an extremely important property for any blood substitute. The longer the product can be stored, the more cost effective it will be. Since there are no products yet available for clinical use, it is not possible to evaluate their stability. Lyophilization is feasible (Greenburg *et al.*, 1977; DeVenuto *et al.*, 1979), although the temperature of storage is important.

The main deterioration of stored hemoglobin is its oxidation to methemoglobin. This reaction is complex, and its rate depends on the specific chemical modification, the ionic environment, the formulation, and the environmental conditions. Oxidation reactions in normal human hemoglobin are still not understood completely, so such studies are necessarily empirical. However, it is possible that by the systematic study of oxidation mechanisms in a variety of modified and mutant hemoglobins, new insights into the reaction will be achieved.

EDITOR'S SUMMARY

The extraordinary biological success of humans is a consequence of extreme flexibility with regard to basic mechanisms such as oxygen transport. The entire system, from uptake of oxygen from the atmosphere in the lung to its delivery to mitochondria in the microcirculation, must be able to cope with all conditions from high altitudes to disease and extreme exertion; if it were any other way, humans would have been extinct millennia ago.

Flexibility has a price: any mechanism that serves such an extreme variety of conditions may not be optimal for any one particular set of conditions. Hence it is not surprising that cell-free oxygen carriers can be designed for specific applications where they may be more effective than red blood cells, and in some instances strikingly so.

This chapter reviews very briefly some of the more obvious clinical applications for blood substitutes, and presents speculation about what the properties of the solutions for these applications might be. The main properties of hemoglobin solutions, for example, that can be easily manipulated are the oxygen affinity, oncotic pressure, viscosity, and molecular size and surface chemistry, which determine intravascular retention time. It should be possible to combine the main beneficial features of crystalloids, colloids and oxygen carriers into one product that would be optimal for the immediate restitution of tissue oxygenation and therefore aerobic metabolism after acute hemorrhagic shock.

Although no product is currently approved for clinical use by any regulatory body in Europe, the US or Japan, it is proposed that these properties can be matched to any proposed clinical application. It is predicted that eventually an array of products might be available, each with unique properties. It is unlikely that an artificial solution that is as universally useful as red blood cells will be developed anytime in the near future.

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Chapter 11

Crystalloid Solutions

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INTRODUCTION

Crystalloid intravenous fluids, which include solutions containing small molecular weight solutes such as sodium, chloride and glucose, are the most common type of fluid used to replace blood in the United States. Colloid solutions, which include solutions containing larger molecular weight solutes such as albumin or hetastarch, are used more commonly in Europe. In crystalloid solutions, the colloid osmotic pressure (osmotic pressure contributed by larger molecules) is by definition zero. In most patients, substitution of crystalloid fluid for blood proceeds without incident because the volume of blood lost is small and the limitations of crystalloid as a blood replacement are not important. However, in patients who suffer extensive blood loss, the characteristics of crystalloid fluids must be understood if important complications are to be avoided.

Exhaustive research has failed to establish the superiority of either colloid-containing or crystalloid fluids for blood replacement or for other purposes. Systematic reviews of available comparisons (many of which were performed years ago) of colloid versus crystalloid (Schierhout and Roberts, 1998) and albumin versus crystalloid (Cochrane Injuries Group Albumin Reviewers, 1998) suggest increased mortality associated with colloid use, although other systematic reviews find no difference after adjusting for characteristics of studies that were included (Wilkes and Navickis, 2001). Other reviews suggest that crystalloid may be superior in multiply traumatized patients (Velanovich, 1989; Choi *et al.*, 1999).

The use of crystalloids for blood replacement was first anticipated in the nineteenth century. In the 1880s, Ringer (1880) observed that salts of sodium, potassium, calcium and chloride in precise proportions and concentrations were necessary for cellular function; these observations led to the later development of balanced salt solutions. In 1899, Crile (1947) resuscitated animals subjected to hemorrhagic shock with warm intravenous infusions of saline and further refined the concept of treatment of shock with crystalloids. During World War I, battle casualties were treated with combinations of colloid and salt solutions. The effects of saline solutions alone were thought to be transient. Blalock, who first categorized shock as hemorrhagic, cardiogenic, neurogenic or septic (Blalock, 1930), also demonstrated that tissue trauma resulted in the loss of extracellular fluid (Blalock, 1940). Hartmann, in the early 1930s, added sodium lactate to saline to avoid hyperchloremic acidosis that he observed when treating children with saline for infantile diarrhea; ultimately, this innovation resulted in lactated Ringer's (RL) or Hartmann's solution (Hartmann and Senna, 1932; Hartmann, 1934).

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World War II prompted renewed insights in hypovolemic shock, which by then could be treated in the field with plasma and blood due to improved logistics (Cournand *et al*., 1943). During this interval, perioperative fluid management was 'salt restrictive,' based on the observations that most patients lost little fluid during elective surgery and that the physiologic stress response resulted in water and salt retention (Coller *et al*., 1944; Moore and Ball, 1952; Hardaway, 1992). In the 1960s, Shires *et al*. (1960, 1961, 1964) documented the necessity of adding substantial volumes of crystalloids to whole blood and plasma to achieve successful resuscitation from hemorrhagic shock. Shires *et al*. (1961) further extended the concept that extracellular fluid volume decreased also during major surgery. As a consequence of these studies, infusion of large amounts of crystalloids became the standard of care in the Vietnam conflict and was associated with a reduction in the rate of renal failure. However, as increased crystalloid volumes became more prevalent, the 'shock lung' syndrome, now called the acute respiratory distress syndrome (ARDS), was first recognized (Knight, 1973; Hirsch, 1987). Subsequently, aggressive fluid resuscitation with crystalloids gained increasing acceptance despite clear distinctions between extensive trauma and elective surgical procedures (Moore and Shires, 1967).

The purpose of this chapter is to review basic physiology relating to fluid distribution as it pertains to blood replacement with crystalloid fluids.

PHYSIOLOGICAL PRINCIPLES

The activity (concentration) of particles in a solution is expressed as osmolarity or osmolality. Osmolarity is expressed in osmoles per liter of solvent (Osm/l); osmolality is expressed as mOsm/kg. Sodium is the principal osmotically active constituent of extracellular fluid. For example, the osmotic activity of 0.9 per cent saline, which contains 154 mEq of sodium ions and 154 mEq of chloride ions per liter, is 308 mOsm/g. Intravenous fluids differ in osmolality and tonicity.

Osmotic activity can also be expressed in terms of *osmotic pressure*, which is calculated from osmolality through the following equation:

$$\Delta \text{ Osmotic pressure (mmHg)} = 19.3 \times \Delta \text{ osmolality (mOsm/kg)} \quad (11.1)$$

Hyperosmolarity is present when the number of osmotically active particles, such as urea, glucose or sodium, is high. When the hyperosmolarity is associated with redistribution of water from the intracellular to the extracellular volume, as occurs with hypernatremia, the resulting condition is termed *hypertonicity.* Thus, the osmolalities of various intravenous fluids in comparison to normal serum osmolality are referred to as isotonic, hypotonic or hypertonic.

DISTRIBUTION VOLUMES OF WATER, SODIUM AND PROTEIN

Water, which accounts for 60 per cent of total body weight in thin, young humans, is contained in three compartments: intracellular (40 per cent), interstitial (16 per cent) and intravascular (4 per cent). The latter two form the extracellular space. Therefore, in a 70-kg adult, total body water = 42 l, intracellular volume = 28 l and extracellular volume = 14 l, of which four-fifths (approximately 11.2 l) is interstitial and one-fifth (approximately 2.8 l) is plasma volume. Sodium is the dominant extracellular cation, 140 mEq/l, whereas potassium is the dominant intracellular cation, 150 mEq/l.

Colloid osmotic pressure (π), though a small component of total osmotic pressure, plays an important role in maintaining the balance between intravascular and interstitial volume. The Starling equilibrium, which mathematically summarizes the forces governing the flow of fluid out of blood vessels into surrounding tissues, states that:

$$Q_f = K_f[(P_c - P_i) - \sigma_d(\pi_c - \pi_i)] \quad (11.2)$$

where Q_f is the total fluid flux out of capillaries and K_f is the filtration coefficient (the product of the membrane conductance and the membrane surface area), P_c is intravascular hydrostatic pressure, P_i is interstitial hydrostatic pressure, π_c is colloid osmotic pressure within the vasculature, π_i is interstitial colloid osmotic pressure gradient across the vessel wall, and σ_d is the oncotic reflection coefficient, the tendency of a membrane to impede the passage of oncotically active particles (Starling, 1896). A σ_d of 0 indicates a membrane that is totally permeable to protein while a σ_d of 1 indicates a membrane that completely prevents protein diffusion. The colloid osmotic pressure gradient depends on

the ratio of lymph to plasma protein, normally 0.65–0.75 (Lai-Fook, 1986). In the lung, σ_d is normally high (0.8–1.0) (Parker *et al.*, 1989) but can decrease in conditions such as ARDS.

The usual distribution of sodium and water suggests the likely distribution volumes of intravenously administered crystalloids and colloids. Sodium-free water will be distributed across total body water. Therefore, 5 per cent dextrose in water, after cellular uptake of the dextrose, will distribute such that the ratio of plasma volume expansion to total body water expansion is 2.8 : 42 (i.e., approximately one-fifteenth will remain intravascular). Solutions containing sodium in physiologic concentrations will be distributed almost exclusively within the extracellular space (approximately one-fifth of which is plasma volume). Therefore, 0.9 per cent saline will distribute such that the ratio of plasma volume expansion to total extracellular volume expansion is 2.8 : 14 (i.e., about one-fifth of infused fluid will remain intravascular). Intravenously administered solutions, in which colloid osmotic pressure is equal to or above plasma colloid osmotic pressure, will remain within the vascular space.

However, these estimates are at best static; they do not consider dynamic conditions in a clinical setting and do not account for renal excretion of infused fluids. In critically ill surgical patients, Hauser *et al.* (1980) showed that infusion of 1 l of lactated Ringer's solution expanded plasma volume by only 190 ml. Lamke and Liljedahl (1976) measured postoperative plasma volumes after infusion of various volume expanders. The average loss of plasma volume during surgery was 250 ml, which was difficult to replace fully with saline infusion.

To more precisely quantify plasma volume expansion produced by infused fluids, several methods have been used, including isotope dilution (Moore *et al.*, 1966) and indocyanine green measurements of plasma volume (Krejcie *et al.*, 1994). However, such methods require extensive intervals of equilibration and are not suitable for rapidly changing clinical situations. Clinical situations in which more quantitative information would be useful include fluid deprivation, hypoproteinemia, anesthesia and surgery (Svensen *et al.*, 1999; Brauer *et al.*, 2002; Connolly *et al.*, 2003), hemorrhage and sepsis (Anderson *et al.*, 1979; Godsoe *et al.*, 1988; Svensen *et al.*, 1997). Disease-induced changes in protein concentration and membrane permeability are particularly important.

KINETIC DISTRIBUTION VOLUMES OF FLUIDS

During and after infusion of crystalloid fluids, the influence of infusion on plasma volume can be estimated using the mass balance approach or the volume kinetic approach. Both quantify the volume effect of an infused fluid by measuring changes in blood hemoglobin concentration ([*Hb*]) following infusion, and both acknowledge that volume status is continuously changing during and after fluid infusion (Figure 11.1). Both assume that hemoglobin concentration is a nearly ideal tracer of changes in plasma volume because hemoglobin is restricted to the intravascular compartment and, in the absence of bleeding or transfusion, is unchanging over the usual course of a study.

When using hemoglobin concentration to estimate plasma volume changes, the baseline blood volume is estimated at baseline (time 0) via the Equation 11.3 (Nadler *et al.*, 1962):

$$BV_0(L) = \begin{Bmatrix} 0.03219\ \text{weight (kg)} \\ +0.3669\ \text{height}^3(m) \\ +0.6041 \end{Bmatrix} \qquad (11.3)$$

The blood volume change at any time (t) can then be calculated as in Equation 11.4:

$$BV(t) = BV_0\left(\frac{Hb_0}{Hb(t)}\right) - BV_0 \qquad (11.4)$$

The amount of fluid retained in the blood (efficacy of the fluid) is given by Equation 11.5:

$$\text{Fluid retained (\%)} = 100 \times \frac{BV(t)}{\text{Infused volume}} \qquad (11.5)$$

The difference between infused fluid volume and the sum of urinary output and blood volume expansion can be used to estimate expansion of the interstitial fluid space (Tollofsrud *et al.*, 2001). This mass balance approach clarifies the temporal effects of crystalloid infusions and is more informative than previous static estimates because it introduces the importance of time after infusion to estimates of volume effects.

Although the mass balance approach is better than static estimates based on distribution volumes, there are several potential limitations of using hemoglobin concentration dilution to estimate plasma volume expansion. Because of differences between hemoglobin concentration in large and small vessels (Chaplin *et al.*, 1953), the hematocrit factor, which is approximately 0.9,

should be used to adjust calculations. In some experimental animals such as dogs, cats and sheep, the hemoglobin concentration may be raised by stress-induced release of erythrocytes stored in the splenic reservoir. In contrast, this effect in humans is negligible (Ebert and Stead, 1941). As fluid is infused, the rate of equilibration of hemoglobin concentration may vary in tissues that are highly perfused versus those that are less well perfused.

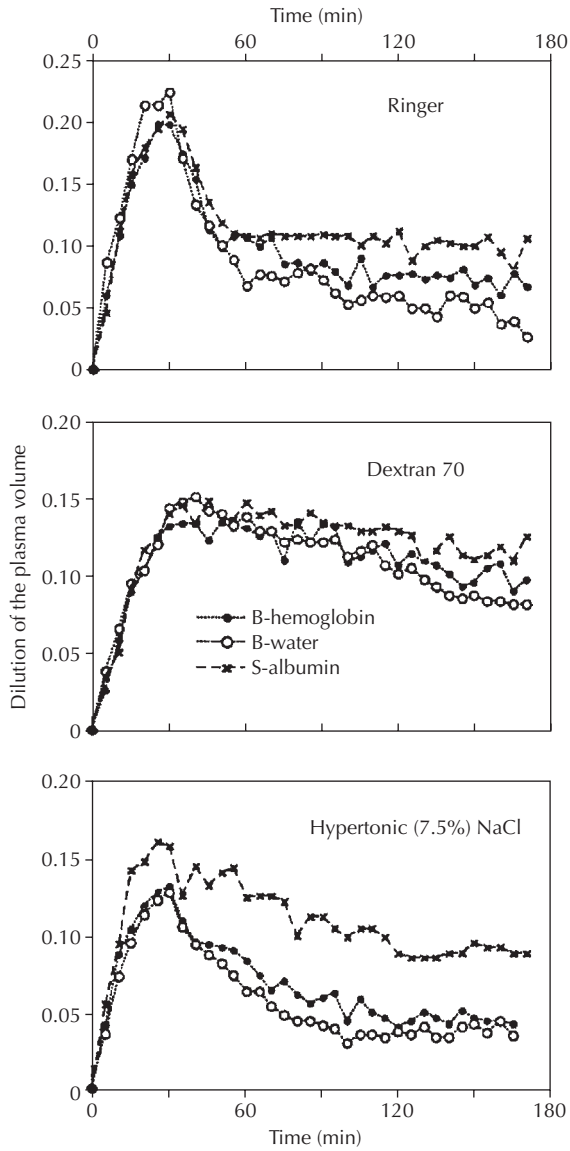

Figure 11.1 The blood hemoglobin (B-hemoglobin), blood water (B-water), and serum albumin (S-albumin) concentrations used as markers to indicate changes in the dilution of the plasma volume during intravenous infusion of 25 ml/kg of Ringer's solution (upper), 5 ml/kg of dextran 70 (middle), and 3 ml/kg of 7.5 per cent saline (lower) over 30 minutes in eight male volunteers. Data are mean values, and correction for blood sampling was not made. (Reproduced from Svensen and Hahn, 1997, with permission.)

Kinetic analysis of the curves describing changes in hemoglobin concentration during and after fluid infusion can be used to clarify the post-infusion effects on plasma volume and clearance of fluid (Figure 11.2). Such an analysis serves the same purpose as pharmacokinetic analysis of drug concentrations (i.e., estimation of peak concentration and rate of elimination from plasma). For these purposes, repeated measurement of hemoglobin concentration provides similar estimates of volumes of distribution and clearance rates as the more tedious and much less practical measurement of blood water concentration (Svensen and Hahn, 1997). Analogous to pharmacokinetic concepts, infused fluid is said to expand one or several *functional* body fluid spaces instead of being distributed between *physiological* fluid spaces. These functional spaces have a baseline 'target volume' that compensatory mechanisms in the body strive to regain after fluid has been administered (Svensen and Hahn, 2000) and after fluid has been lost.

For kinetic analysis, dilution data are fitted to the solutions of differential equations derived from a kinetic model to reflect a simplified version of physiologic processes. The parameters in the model, such as the volume of distribution and the rate of elimination, are then estimated by non-linear least-squares regression. When the parameters in the kinetic model have been estimated, the infused fluid is considered to expand a single body fluid space v, which the body strives to maintain at the target volume V (Figure 11.3).

Elimination of fluid occurs by baseline urinary excretion and evaporation (k_b), and by a dilution-dependent mechanism governed by a constant (k_r). The volume changes in the one-volume model are given by the following differential equation:

$$\frac{dv}{dt} = k_i - k_b - k_r \frac{(v - V)}{V} \qquad (11.6)$$

In a two-volume model, the infused fluid is considered to expand both a single central and a more peripheral body fluid space called v_1 and v_2, respectively (Figure 11.3). In the one-volume model, elimination of fluid occurs from v_1 by two mechanisms, k_b and k_r. The expanded space v_1 communicates with v_2 and the net rate of volume

equilibration between them is proportional by a constant (k_t) to their relative differences in dilution. The volume changes in the two-volume-model are given by the following differential equations:

$$\frac{dv_1}{dt} = k_i - k_b - k_r \frac{(v_1 - V_1)}{V_1} - k_t \left[\frac{(v_1 - V_1)}{V_1} - \frac{(v_2 - V_2)}{V_2} \right] \quad (11.7)$$

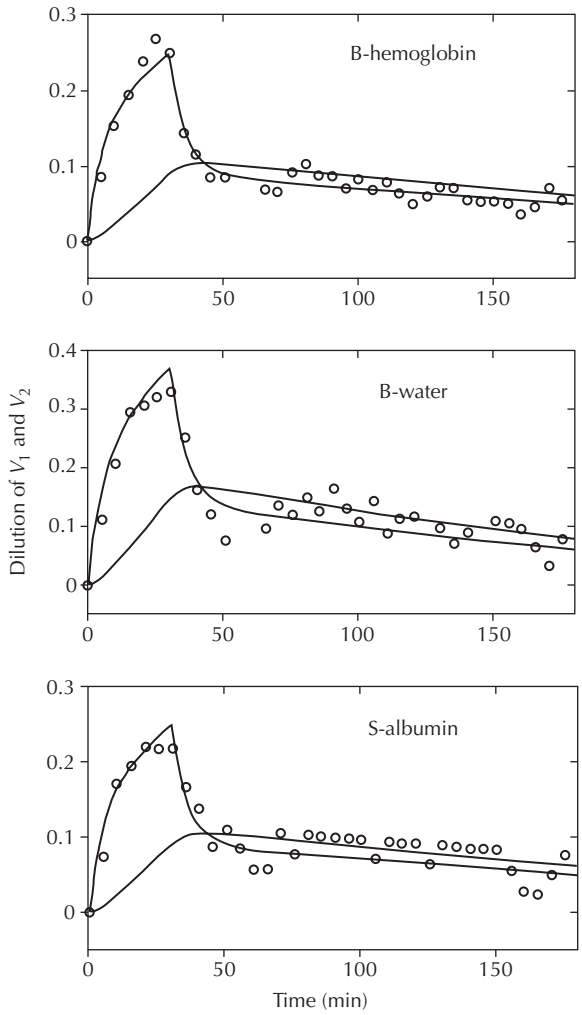

Figure 11.2 Representative graphic output of the volume kinetic analysis in one male volunteer in whom the blood hemoglobin (B-hemoglobin; upper), blood water (B-water; middle), and serum albumin (S-albumin; lower) concentrations were used as markers of dilution during and after an intravenous infusion of Ringer's solution. The measured dilution (circles) and the model-predicted dilution–time curve for the primary fluid space (upper line during infusion) and the secondary fluid space are shown. (Reproduced from Svensen and Hahn, 1997, with permission.)

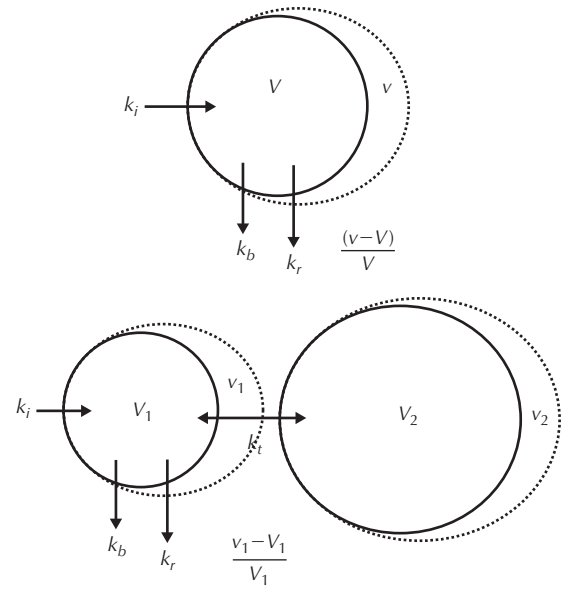

Figure 11.3 Schematic drawing of the kinetic model used to calculate the size of the body fluid spaces expanded by intravenous infusions of fluid in humans. Data are fitted to a one-volume or two-volume-of-fluid-space (VOFS) model. The assumptions underlying the one-compartment VOFS model (top) are as follows: (1) during fluid infusion, fluid enters an expandable space of volume v at a constant rate k_i; (2) the expandable fluid space has a target volume V, which the body strives to maintain; (3) volume v changes by fluid being eliminated from the fluid space at a basal rate, k_b (perspiration and basal diuresis), and at a controlled rate. The controlled rate is proportional by a constant k_r to the relative deviation of v from the target volume V. The assumptions behind the two-compartment VOFS model (bottom) are similar: (1) during fluid infusion, fluid enters an expandable space of volume v_1 at a constant rate k_i; (2) there is a secondary expandable fluid space of volume v_2 exchanging fluid with the primary fluid space; (3) volume v_1 changes through exchange with the secondary fluid space and as a result of fluid being eliminated from the primary fluid space at a basal rate, k_b (perspiration and basal diuresis), and at a controlled rate; (4) the primary and secondary fluid spaces have target volumes V_1 and V_2, which the system strives to maintain by acting upon the controlled elimination mechanism k_r, which is proportional to the relative deviation from the target volume of the primary fluid space, and by acting on the fluid exchange mechanism; (5) the net rate of fluid exchange between the two spaces is proportional to the difference in relative deviations from the target volumes by a constant k_t. (Reproduced from Svensen and Hahn, 1997, with permission.)

$$\frac{dv_2}{dt} = k_t \left[\frac{(v_1 - V_1)}{V_1} - \frac{(v_2 - V_2)}{V_2} \right] \qquad (11.8)$$

The dilution–time profiles, sometimes augmented by measurement of urinary excretion, are entered into a computer that fits the solutions to the differential equations describing the fluid shifts in these kinetic models to the data (Svensen and Hahn, 1997). Results are the optimal values for the unknown parameters in the models together with the uncertainties of each estimate. The unknown parameters in the one-volume model, are V and k_r, while in the two-volume model the corresponding unknown parameters are V_1, V_2, k_t, and k_r.

Analysis of the dilution–time profile according to the volume kinetic models has proven to be a useful tool in the study of the effects of fluid therapy. For a crystalloid fluid such as buffered Ringer's solution, the one-volume kinetic model typically applies when urinary excretion is prompt. Since distribution between V_1 and V_2 requires as much as 30 minutes to be completed, peripheral edema does not develop (i.e., fluid does not translocate from V_1 to V_2) if the renal excretion is effective (k_r is high). In contrast, the two-volume model normally applies in dehydrated subjects and during surgery, probably because of physiologic responses that tend to conserve extracellular volume.

CRYSTALLOID SOLUTIONS

A crystalloid solution contains small particles that are composed of low molecular weight solutes (<30 000 D) that can be either ionic (e.g. Na^+, Cl^-) or non-ionic (e.g. glucose, mannitol). Crystalloid fluids are inexpensive compared with blood products and artificial colloids. The contents of a number of commonly used crystalloids are listed in Table 11.1. The systemic distribution of crystalloids is dependent on the amount of sodium in the solution. Solutions that contain approximately isotonic sodium concentrations (e.g. 0.9 per cent saline, lactated Ringer's solution) will remain largely within the extracellular fluid space.

In clinical practice, hypotonic, isotonic and hypertonic solutions are used for various indications, although isotonic solutions are most often selected to replace blood lost by hemorrhage. Hypotonic solutions (e.g. 0.45 per cent saline) contain sodium-free water which, when infused rapidly in large volumes, can reduce plasma osmolality (i.e., reduce plasma sodium concentration). The primary indication for infusion of hypotonic fluids is hypertonic dehydration (i.e., hypernatremia due to deficits of both sodium and water but relatively more water). Hypotonic solutions should also be used with care in patients with traumatic brain injury, since they can increase cerebral water and raise intracranial pressure (ICP).

The most commonly used isotonic fluids are lactated Ringer's solution (lactated, RL or acetated, RA) or 0.9 per cent saline. RL or RA solutions are mildly hypotonic when compared with plasma, but are still regarded as isotonic. A theoretical advantage of acetate-containing solutions is that acetate can be metabolized by all tissue cells, unlike lactate, which requires renal or hepatic metabolism.

Table 11.1 Physicochemical properties of common crystalloids

Fluid	Osmolality (mmol/kg)	pH	Na^+ (mEq/l)	K^+ (mEq/l)	HCO_3^- (mEq/l)	Cl^- (mEq/l)	Ca^{++} (mEq/l)	Dextrose (g/dl)
0.9% Saline	308	5	154	0	0	154	0	
Hartmann's solution	280	6.5	131	5	29	111	2	
Lactated Ringer's	273	6.5	130	4	28*	109	3	
Acetated Ringer's	270	6	130	4	30*	110	2	
5% Dextrose in water	253	4	0	0	0	0	0	50
5% Dextrose in 0.45% saline	505	4	77	0	0	77	0	
2.5% Dextrose in saline	270	6	70	0	25	45	0	25
Plasmalyte A	294	7.4	140	5	50	98	0	
3% Saline	1026		513			513	0	
7.5% Saline	2400		1250			1250		

*Concentrations of lactate or acetate that are metabolized to bicarbonate.

Hypertonic saline solutions have not yet been incorporated into perioperative fluid management, although there are many promising studies addressing the volume effects and anti-inflammatory properties during surgical procedures (Christ *et al.*, 1997; Junger *et al.*, 1997; Kreimeier *et al.*, 1997). Saline solutions with sodium concentrations ranging from 1.5 per cent to 30 per cent have been used in a variety of circumstances. In general, hypertonic crystalloid solutions cause greater plasma volume expansion than do similar volumes of isotonic solutions, but in the pre-hospital and military settings (Committee on Fluid Resuscitation for Combat Casualties, 1999) they have been introduced, and they are commonly used in several countries other than the US (Wade *et al.*, 1997a, 1997b). Perhaps their best theoretical application is in the resuscitation of trauma patients who have both severe systemic hemorrhage and traumatic brain injury (Vassar *et al.*, 1991, 1993). Nevertheless, a recent randomized controlled trial failed to show any benefit of hypertonic saline resuscitation in hypotensive, brain-injured trauma patients (Cooper *et al.*, 2004) (Figure 11.4).

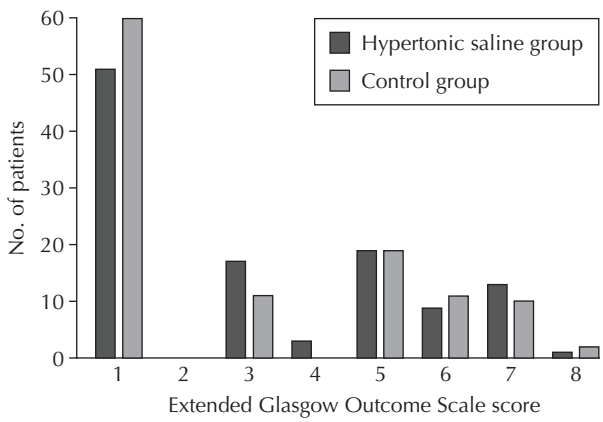

Figure 11.4 Outcome of hypotensive, traumatically brain-injured patients randomized to 250 ml of 7.5% hypertonic saline or lactated Ringer's solution for initial resuscitation. The extended Glasgow Outcome Scale is an eight-point scale whereby 1 indicates dead; 2, vegetative state; 3, lower severe disability; 4 upper severe disability; 5, lower moderate disability; 6, upper moderate disability; 7 lower good recovery; and 8, upper good recovery. In the hypertonic saline group, 62 of 113 patients survived to 6 months; in the lactated RL group (control), 53 of 113 patients survived to 6 months. (Reproduced from Cooper *et al.*, 2004, with permission.)

USE OF CRYSTALLOID FLUIDS IN SURGICAL PROCEDURES

The strategy of using crystalloids without colloid for replacement of fluid losses during elective surgical procedures continues to be controversial. In experimental animals subjected to hemorrhagic shock (Shires *et al.*, 1960, 1961) and traumatized patients (Shires *et al.*, 1961, 1964), Shires *et al.* described substantial decreases in 'functional' extracellular volume, which they explained as sequestration within traumatized tissue, bowel and peritoneum. They recommended replacement with generous quantities of isotonic crystalloids. These results led to substantially increased volumes of fluid replacement for hemorrhagic shock and elective surgical procedures (Moore and Shires, 1967; Cohn *et al.*, 1970; Glaser *et al.*, 1995; Lang *et al.*, 2001; Holte *et al.*, 2002).

Physiologic responses to perioperative crystalloid loading appear to be complex. The Starling equilibrium describes a simple relationship between capillary hydrostatic pressure and colloid osmotic pressure. However, multiple factors also influence net movement of fluid from capillaries from plasma into interstitium. Dilution of plasma colloid osmotic pressure by infusion of an isotonic crystalloid will increase fluid flux into the interstitium, which in turn will dilute interstitial colloid oncotic pressure and partially restore the gradient between plasma and interstitial colloid osmotic pressure. As fluid flux into the interstitium increases, lymphatic flow increases, which returns some interstitial protein to the vascular space (Drucker *et al.*, 1981). Lung lymph flow has the capacity to increase vastly when challenged by lower plasma oncotic forces (Zarins *et al.*, 1978; Kramer *et al.*, 1983, Wareing *et al.*, 1989). During hemorrhage, constriction of precapillary sphincters reduces capillary hydrostatic pressure, which promotes movement of fluid into the capillaries and partially restore intravascular volume. Prolonged or severe hemorrhage will also generate insulin resistance, thereby causing hyperglycemia, which increases osmotic reabsorption of fluid into capillaries (Oberg, 1964; Lundvall and Hillman, 1978; Haljamae, 1985; Lundvall and Länne, 1989). When the capillary endothelium is damaged by inflammatory responses, as in sepsis or prolonged hemorrhagic shock, increased permeability to proteins can largely eliminate the colloid osmotic pressure gradient, thereby

resulting in transcapillary fluid flux that is determined largely by capillary hydrostatic pressure.

The importance of postoperative crystalloid fluid accumulation is controversial. Extravascular accumulation of infused crystalloids is evident in highly compliant tissues such as skin and connective tissue, but also occurs in parenchymal organs such as the lungs (Haljamae, 1999). Fluid accumulation in the lungs represents a particular concern. Based on a 1-year retrospective review of patients undergoing major surgery at two university medical centers in the US, Arieff *et al*. (1999) projected an annual incidence of 8000–74 000 cases of postoperative pulmonary edema, including many patients in which there were no apparent risk factors other than infusion of large volumes of crystalloid solutions.

A second area of clinical concern is inhibition of gastrointestinal motility and delayed healing of anastomoses (Hauser *et al*., 1980). Brandstrup *et al*. (2003), in a recent randomized clinical trial of colorectal surgical patients, compared a severely restricted fluid regimen versus a standard fluid regimen and showed significantly higher morbidity in the group given larger amounts of fluid. However, these results specifically apply to patients undergoing colonic surgery. Holte *et al*. (2004) randomized patients undergoing laparoscopic cholecystectomy to receive either 15 ml/kg or 4 ml/kg of RL intraoperatively, and found a significant improvement in postoperative pulmonary function, exercise capacity and overall well-being in the group receiving the higher fluid volume. Clearly, further studies are necessary to clarify the interactions between perioperative fluid infusion, surgical procedure and patient demographic factors.

USE OF CRYSTALLOID FLUIDS FOR REPLACEMENT OF BLOOD LOSS

An important question relating to blood replacement with isotonic crystalloids is the ratio of crystalloid that is required relative to the volume of shed blood. The conventional recommendation is 3 : 1, although there is no apparent physiologic rationale for that ratio. Previous hemorrhage does influence plasma volume expansion after crystalloid infusion. Drobin and Hahn (1999) demonstrated greater plasma dilution and longer preservation of plasma dilution in conscious volunteers who had hemorrhaged 450 ml or 900 ml before infusion of 25 ml/kg (1750 ml per 70-kg) of isotonic crystalloid over 30 minutes. However, plasma dilution was greater in part because baseline plasma volume was reduced by hemorrhage; therefore, peak estimated plasma volume expansion produced by the infusions was similar – approximately 800 ml under all three conditions. Retention of plasma volume was approximately twice as great within the first 2 hours after either volume of hemorrhage in comparison to the normovolemic state.

Experimental data suggest that the ratio of 3 : 1 underestimates the volume of crystalloid necessary to replace shed blood if hemorrhage is severe. Michalski *et al*. (1968) sequentially removed 500 ml of blood in lightly halothane-anesthetized dogs while rapidly infusing warmed RL to restore mean arterial blood pressure, then repeated the procedure after 20-minute stabilization intervals until hematocrit was less than 10 per cent. Approximately three times as much RL was infused as blood was withdrawn; however, continued rapid fluid infusion was required for 60 minutes until shed blood was returned. More importantly, the investigators failed to restore mean arterial pressure and the dogs developed metabolic acidosis. Although this study did not define the ratio of isotonic crystalloid to shed blood necessary to restore normal perfusion, the data suggested that a 3 : 1 ratio was insufficient. Subsequently, Cervera and Moss (1975) used a 3 : 1 ratio of LR to replace shed blood in anesthetized dogs. When 65 per cent of the original red cell mass had been removed, the dogs were in frank hypovolemic shock, had a 30 per cent reduction in blood volume and required an additional 4.7 ml/kg of RL to restore hemodynamic stability. From these two experimental studies, the ratio of isotonic crystalloid to shed blood that restores hemodynamic stability appears to be considerably greater than 3 : 1 and perhaps exceeds 7 : 1.

OTHER EFFECTS OF CRYSTALLOID INFUSION

Trauma, surgery and hemorrhage stimulate the immune system. Trauma with hemorrhagic shock activates neutrophils, and stimulates neutrophil adherence and migration. Although these responses are necessary to resist infection in traumatized tissue, excessive inflammatory responses can lead to ARDS and multiorgan failure.

Infusion of LR, in comparison to infusion of blood or hypertonic saline, stimulated a burst of neutrophil activity in rats with hemorrhagic shock (Rhee *et al.*, 1998, 2000) and was also associated with apoptosis in the gut (Deb *et al.*, 1999). These studies suggest that the choice of resuscitation fluids can influence the subsequent immune responses. However, in rats with massive hemorrhage, LR improved survival in comparison to 0.9 per cent saline (Healey *et al.*, 1998).

When large amounts of 0.9 per cent saline are given perioperatively, hyperchloremic metabolic acidosis is a predictable result (Scheingraber *et al.*, 1999; Waters *et al.*, 1999; Wilkes *et al.*, 2001; Stephens and Mythen, 2003). Although controversy continues regarding whether hyperchloremic metabolic acidosis causes adverse clinical consequences (Waters *et al.*, 2001), limited evidence suggests an association of saline-induced hyperchloremic metabolic acidosis with coagulation defects, impaired urinary output (Reid *et al.*, 2003), objective and subjective central nervous system dysfunction (Williams *et al.*, 1999) and subjective abdominal discomfort (Williams *et al.*, 1999). In contrast, lactate and acetate in Ringer's solutions are metabolized to bicarbonate and are associated with a mild postoperative metabolic alkalosis.

Because the normal blood–brain barrier is highly impermeable to sodium, small changes in serum sodium exert greater osmotic pressure gradients across the cerebral capillary bed than do relatively large changes in serum protein concentrations (Zornow *et al.*,1987). For instance, an increase of 2 mEq/l in serum sodium would increase osmolality by 4 mOsm/kg, or 79.2 mmHg of osmotic pressure (Table 11.2). In contrast, the osmotic pressure exerted by a normal serum protein concentration across the blood–brain barrier is only approximately 23 mmHg.

The effects of changes of serum sodium on brain water or ICP have been extensively studied in animals with normal brains, in experimental models of brain injury, and in humans. Because the blood–brain barrier enhances the influence on brain water of changes in serum sodium (Zornow *et al.*, 1987), hypotonic solutions (including RL) are more likely to increase brain water content than 0.9 per cent saline or colloids dissolved in 0.9 per cent saline. Hypertonic sodium solutions acutely reduce brain water and therefore tend to reduce ICP. In a double-blind, crossover study in head-injured children, 3.0 per cent saline decreased ICP significantly, whereas 0.9 per cent saline had no effect (Fisher *et al.*, 1992). However, other experimental data also suggest the possibility of rebound intracranial hypertension after hypertonic resuscitation from shock and intracranial hypertension (Prough *et al.*, 1999). As noted previously, hypertonic solutions have not improved outcome in hypotensive, traumatically brain-injured patients (Cooper *et al.*, 2004).

Experimental models that examine the acute effects of rapid administration of fluids of varying tonicity usually demonstrate differences in brain water; those that look at slower, 'maintenance' rates of administration usually find negligible differences. Shapira *et al.* (1992) compared the effects on brain water of a variety of fluids administered to head-injured rats for 18 hours after injury. No differences in brain edema were evident between groups that were fluid restricted and those infused with 25 per cent glucose, 5 per cent dextrose in 0.45 per cent saline, or an isotonic gelatin-based plasma expander.

Both *in vitro* and *in vivo*, crystalloids have been associated with hypercoagulability (Boldt *et al.*, 2002; Ekseth *et al.*, 2002; Martin *et al.*, 2002; Ruttmann *et al.*, 2002), especially when infusion is rapid (Ruttman *et al.*, 1996, 2002). Consequently, some authors have suggested that colloid fluids could be preferable for perioperative management in patients with vascular diseases (Janvrin *et al.*, 1980).

Table 11.2 Acute effects of changing osmotic pressure in the cerebral capillaries

Osmoles	Osmolality (mOsm/kg)		Osmotic pressure (mmHg)		Osmotic pressure difference (mmHg) (Plasma – IF)
	Plasma	*IF	Plasma	IF	
$[Na^+]$, protein, non-protein	282.6	282.6	5454	5454	0
$[Na^+]$ acutely ↑ 2.0 mEq/l	286.6	282.6	5533.2	5454	79.2
Protein	1.2	0	23	0	23
Protein ↑ × 2	2.4	0	46	0	46

*IF, interstitial fluid.

SUMMARY

Although the overall safety of crystalloid fluids for blood replacement and perioperative fluid management appears to be outstanding, there remain several areas in which further information is necessary. The kinetics of crystalloids may favor interstitial accumulation during perioperative administration, and may be associated with postoperative complications in certain types of surgery such as bowel surgery. The effects of specific crystalloids on immune function, metabolic status and coagulation are potentially important and require continued study.

EDITOR'S SUMMARY

Crystalloid (salt) solutions are the cornerstone of clinical volume replacement, either during surgery or to treat traumatic blood loss. In contrast to blood transfusion, crystalloids are distributed significantly in the extravascular spaces, and the rates of distribution after infusion are not easily predictable. This chapter presents a theoretical basis for this phenomenon and explains why adequate replacement of blood losses with crystalloid requires infusion of between three and seven times the volume of loss. Infusion of such large volumes can have undesired consequences, such as hypercoagulation, acidosis and tissue edema, particularly in the lung and gastrointestinal tract. It is important to understand the physiologic changes that follow infusion of these solutions. Furthermore, when blood substitutes are available, they will be used in combination with crystalloids. More research is needed to understand the combined effects of these two volume expanders.

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Chapter 12

Hemoglobin-Based Oxygen Carriers as Resuscitative Solutions for Trauma and Combat Casualty Care

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INTRODUCTION

Immense scientific and commercial efforts continue to be undertaken to develop a safe and effective synthetic oxygen-carrying solution that can be used in place of blood or packed red blood cells (RBCs). The greatest progress has been made in the development of modified hemoglobin solutions, commonly called hemoglobin-based oxygen carriers (HBOCs). The goal has been to produce a safe and effective HBOC with the functionality of packed red blood cells and without the significant limitations associated with blood (i.e., immune suppression, loss of efficacy with storage, and risk of viral contaminants). Such a product would have a huge market for preoperative and critical care medicine as a replacement for the current blood supply. Further, the hope is that an easily storable product could be used effectively for pre-hospital and battlefield trauma where current fluid resuscitation strategies are lacking in efficacy.

The clinical need and physical characteristics of HBOCs suggest two different roles for the solutions: correction of anemia, and resuscitation of hypovolemic blood loss. This chapter evaluates the potential role of HBOCs as resuscitative fluids; particular focus is placed on the potential use of HBOCs as initial resuscitative solutions for combat casualty care. Specifically, we will discuss current strategies for combat casualty care using existing US Food and Drug Administration (FDA)-approved fluids; analyze HBOC solutions currently under development; and present some of the data from the HBOC clinical trauma trials and animal studies of hemorrhage.

REQUIREMENTS FOR COMBAT CASUALTY CARE

In US trauma centers, large volumes of crystalloids, particularly lactated Ringer's solution (RL), are used for resuscitation of hemorrhage. Since each liter of RL only expands blood volume by 20–30 per cent of infused volume, several liters (3–5 liters infused to replace 1 liter bled) of fluid are required to restore near normal blood volume after a major hemorrhage (Lamke and Liljedahl, 1976; Drobin and Hahn, 1999; Tølløfsrud *et al.*, 2001). Such volumes are logistically untenable for most combat casualty care scenarios. Currently,

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for field resuscitation the US Army uses colloids such as Hextend™, which contains hetastarch and which can reduce volume requirements. One liter of hetastarch can expand blood volume about 1 liter. Hypertonic saline mobilizes cellular water and can also reduce volume needs. However, there is a limit to how much hetastarch can safely be infused, as coagulopathies are known to occur with doses over 20 ml/kg (Konrad *et al.*, 1999). Another strategy employed is use of hypotensive resuscitation, in which the goal or resuscitated endpoint is the restoration of a subnormal blood pressure. The original rationale for using hypotensive resuscitation was to reduce the risk and occurrences of increased bleeding that can occur with rapid restoration of normal pressures and blood flows. Resuscitation studies in anesthetized animal models of uncontrolled hemorrhage (aortotomy or tail ligation) suggest that target mean arterial pressures of 40–60 mmHg are ideal (Stern *et al.*, 1993; Capone *et al.*, 1995). Other studies using conscious animals suggest that target pressures should be above 65 mmHg (Xianren *et al.*, 2003; Rafie *et al.*, 2004). Although the perfect target pressure remains to be determined, there is significant rationale for the use of some form of limited resuscitation. Hypotensive resuscitation is already practiced by some military units using pulse strength and level of consciousness as an approximate measure of blood pressure. Stern (2001) has written an elegant review of hypotensive resuscitation and makes the case for its application, but she cautions against the widespread clinical application of hypotensive resuscitation until randomized clinical trials have been performed.

Employing a hypotensive resuscitation strategy to treat combat casualty care offers the practical benefit of greatly reducing volume needs. Recent research by our group using conscious hemorrhaged sheep suggests significant volume sparing (particularly with LR) when a target mean arterial pressure of 65 mmHg is used (Michell *et al.*, 2003; Vaid *et al.*, 2003; Rafie *et al.*, 2004) (Table 12.1). Volume requirements of all FDA-approved fluids can be reduced when used with a hypotensive strategy. The impact of such a strategy may or may not be beneficial to outcomes. Some animal models show reduced mortality with hypotensive resuscitation strategies, but other more recent animal models suggest that mortality is increased when such strategies are employed. Notably, Sondeen *et al.* (2004) showed a 100 per cent 24-hour survival rate when a large volume of RL was used per Advance Trauma Life Support (ATLS) guidelines, but survival rates were reduced to 33 per cent to 50 per cent when hypotensive strategies were used with either RL or Hextend to treat hemorrhaged swine. Other conscious animal studies of limited resuscitation have suggested that such strategies can be less efficacious than standard ATLS with respect to survival, and that these methods do not adequately normalize lactate levels (Xianren *et al.*, 2003; Sondeen *et al.*, 2004). Our group reported some deaths and higher lactate levels when hypotensive resuscitation was used with FDA-approved fluids, including RL, Hextend, or 3 per cent NaCl (Michell *et al.*, 2003; Vaid *et al.*, 2003; Rafie *et al.*, 2004).

The rationale for a hypotensive strategy was supported by two clinical trials in which delaying fluid resuscitation or limiting the amount of fluid infused produced outcomes comparable to or better than those resulting from standard of care ATLS resuscitation (Bickell *et al.*, 1994; Dutton *et al.*, 2002). Bickell *et al.* (1994) showed that delaying fluid therapy until surgery resulted in a higher survival percentage in hypotensive patients with penetrating trauma. Blood pressures increased with aggressive standard ATLS resuscitation from 58 ± 35 to 79 ± 46, whereas in the delayed fluid group blood pressures were increased almost to the same levels (from 59 ± 34 to 72 ± 42). Dutton *et al.* (2002) resuscitated to target endpoints of either 100 mmHg systolic or 70 mmHg systolic, and found no difference in survival between hypotensive resuscitation and standard of care ATLS. However, both groups in the Dutton study achieved systolic pressures above target value during resuscitation to 114 and

Table 12.1 Volumes required to resuscitate to normal and hypotensive targets

Fluid	Normotensive target volume (ml/kg)	Hypotensive target volume (ml/kg)	Volume reduction (%)
Lactated Ringer's (RL)	61.4 ± 11	18.0 ± 6	81
Hextend	19.2 ± 2	11.6 ± 2	40
3% NaCl	19.6 ± 2	13.4 ± 5	32

100 mm Hg, respectively. These small differences in blood pressure between normal and limited resuscitation can be attributed to the normal compensatory mechanisms that allow hypovolemic patients to refill volume and augment cardiovascular function. There is a discrepancy between outcomes of some recent animal trials, in which increased mortality has been reported with hypotensive resuscitation (Michell *et al.*, 2003; Vaid *et al.*, 2003; Sondeen *et al.*, 2003; Rafie *et al.*, 2004), and the clinical trials in which limited fluids seem to improve survival (Bickell *et al.*, 1994) or have no impact (Dutton *et al.*, 2002). Explanations are likely to be multifactorial. Possible explanations are the rapid transport in urban trauma systems versus the sustained periods of hypotension in animal models (designed to mimic the delay in definitive care) used in Department of Defense-funded studies. Additionally, a transducer and catheter are used to measure blood pressure accurately in animal studies, whereas blood pressure in human patients is measured by a cuff, which is subject to error. Moreover, animal models may employ more severe targets than in patient studies.

The Bickell and Dutton studies suggest that limiting fluid therapy may be safe, and such data seem to support application of limited or hypotensive resuscitation. However, the results of these civilian trauma trials do not necessarily model combat casualty care, where definitive care can be greatly delayed and medical personnel and advanced treatment modalities are limited. Compared with the short duration (i.e., less than 1 hour) of time needed to transport injured patients to civilian urban trauma systems, data from recent military conflicts, such as those in Somalia (Mabry *et al.*, 2000) and Afghanistan, have shown that time taken to transport combat casualties to definitive care stations is often several hours in duration. With the increased availability and use of handheld surface-to-air missiles, such delays will likely remain a component of modern combat casualty care.

Present day civilian as well as military treatment of hemorrhage and trauma is to administer asanguineous fluids, crystalloids or colloids. Resuscitation with asanguineous fluids can restore lost volume and increase cardiac output and oxygen delivery. However, the improvement in oxygen delivery is limited by hemodilution and the reduced oxygen content of arterial blood. HBOCs would seem to be ideal resuscitation solutions, since, as colloids, they should be excellent volume expanders and because they are also able to maintain or correct reduced oxygen content. A pharmacological effect of most HBOCs is vasoconstriction, which would at first seem to contraindicate them as resuscitative fluids. However, vasoconstrictors are being advocated in some resuscitation scenarios, including early resuscitation of severe hemorrhage (Morales *et al.*, 1999) and resuscitation of the combined insult of hemorrhage and head injury (Sanui *et al.*, 2004). We are unaware of any studies that have addressed the values or dangers of using a vasoconstrictor in hypotensive resuscitation.

In theory, HBOCs might be the ideal fluid for combat casualty care and limited fluid strategies. Because HBOCs carry oxygen, are colloids and are vasoconstrictors, they are likely to reduce volume needs when infused to a target blood pressure. The ultimate utility of HBOCs for combat casualty care will depend on both their physical and their physiological characteristics. In the following section, we will review the general properties of HBOCs and the variety of products currently under development.

RELEVANT PROPERTIES OF HBOCs FOR TRAUMA AND COMBAT CASUALTY CARE

The complex challenge of developing an oxygen carrier and the relative availability and familiarity with plasma expanders has focused the development of RBC substitutes almost exclusively on their ability to load and unload oxygen. HBOCs have modified oxygen dissociation curves that can alter oxygen uptake and enhance oxygen unloading in tissues (Vandegriff and Winslow, 1995). Several reviews have focused on the oxygen carrier properties of red blood cell substitutes and their clinical utility as such (Greenberg, 1998; Nucci and Abuchowski, 1998; Kramer and Poli de Figueiredo, 1999; Creteur and Vincent, 2003). However, from the perspective of a resuscitative fluid, the more important aspect of HBOCs is their unique pharmacologic and physical properties in solution, which can impart unexpected effects on colloid osmotic pressure (COP) and volume expansion as well as associated hemodynamic responses (Vandegriff *et al.*, 1997; Lurie *et al.*, 2002).

Volume expansion and oxygen delivery are two desirable properties of a resuscitative fluid. Thus, a high COP and a high concentration of

Table 12.2 Hemoglobin concentration and colloid osmotic pressures of HBOCs with advanced clinical testing

Company	HBOC name	Molecule	Conc. (g/dl)	COP (mmHg)
Northfield Labs	PolyHeme	Polymerized human	10	~28
Biopure	Hemopure	Polymerized bovine	13	26
Hemosol	Hemolink*	Polymerized human	10	26
Curacyte, Inc	PHP-Hb	Conjugated with polyoxyethylene	10	96
Sangart	Hemospan®	Conjugated with PEG	4.4	46
Baxter	HemAssist (DCLHb)*	Crosslinked tetramer	10	34
Somatogen	Optro*	Recombinant tetramer	5	~15

*Development cancelled or trials stopped due to adverse outcomes.

hemoglobin would appear to be ideal properties for a resuscitative fluid. If formulations of free cell hemoglobin tetramers had the same concentration as blood (12–18 g/dl) or packed red blood cells (20–25 g/dl), they would be extremely hyperoncotic. Due to the potential for overexpansion and hemodilution, a hyperoncotic pressure has been considered an undesirable property for a red blood cell substitute. Indeed, all commercial solutions currently in development have a limited Hb concentration. Table 12.2 lists the Hb concentrations and the COPs of most of the HBOCs that are or have been in clinical trials as part of the FDA regulatory process. HBOCs made up of conjugated tetramers (HemAssist, Optro™) are either dilute or hyperoncotic. Hemoglobins with added surface decorations or conjugations (Hemospan®, PHP-Hb) have increased viscosity and COP, even in diluted concentrations. Polymerization of hemoglobin tetramers (PolyHeme™, Hemopure™, Hemolink™) is a strategy used to increase the Hb concentration while minimizing increases in COP. The two HBOCs that have advanced the farthest in clinical trials are both glutaraldehyde polymerized hemoglobins made from human blood (PolyHeme™) and bovine blood (Hemopure) with COPs similar to the 25–28 mmHg found in healthy humans.

Perhaps the most extensively studied and financed HBOC was HemAssist, or diaspirin crosslinked hemoglobin (DCLHb), which dramatically failed in trauma trials. Over 100 animal studies and several trials in volunteers and elective surgery patients suggested that DCLHb had acceptable safety and efficacy. However, when used after large-volume RL as early emergency-room treatment of severely traumatized patients, a significantly increased mortality was observed (Sloan and Koenigsberg, 1999; Sloan *et al.*, 1999). When DCLHb was used as first treatment for pre-hospital care in a European trial, there was no impact on survival (Kerner *et al.*, 2003). There may be the need for new resuscitation regimens for safe and effective use of HBOCs. Subsequent animal studies that mimicked severe trauma and hemorrhage also showed an increase in mortality using DCLHb as opposed to packed red blood cells, particularly when DCLHb was infused along with large-volume crystalloid infusions (Gibson *et al.*, 2002; Maxwell *et al.*, 2002). Vane *et al.* (2002) infused DCLHb in hemorrhaged sheep to correct anemia after major surgery and after normalization of filling pressure with LR and noted depressed cardiac output, several deaths, and hemodynamics consistent with volume overload. The underlying message may be that most animal models and even intraoperative clinical trials do not have the sensitivity to fully evaluate the safety or efficacy of HBOCs in severely injured patients. Pre-hospital or emergency-room use of HBOC may be more challenging than intraoperative use, where skilled anesthesiologists pharmacologically titrate infusion rate and administer drugs to prevent extreme hemodynamic alterations. Because of a variety of safety issues with other red blood cell substitutes, the FDA is likely to be cautious and conservative before granting marketing approval to an HBOC. Thus, a commercially available blood substitute may not be available for some years.

INTRAOPERATIVE TRIALS

Most clinical trials have evaluated HBOCs as intraoperative red blood cell substitutes. Other than Baxter's two trauma trials, there have been

no pre-hospital trials of resuscitation. PolyHeme was evaluated in an intraoperative trial as a red blood cell substitute for trauma surgery. This trial produced a 44 per cent reduction in blood needs as compared with the control group (Gould *et al.*, 1998). Some might suggest that it is a disappointment that a 'blood substitute' reduced blood needs by less than one-half; others might counter that any conservation of blood is significant. Projections are that the need for red blood cells will increase as the number of surgeries and the age of surgical patients both continue to increase. An uncontrolled one-group PolyHeme trial with 171 trauma patients showed better outcomes when the data were compared with historical controls of patients with infused blood (Gould *et al.*, 2002). However, the FDA is likely to require concurrent control groups. Neither of the PolyHeme trials led to FDA approval. Unfortunately, adverse events have been reported in most clinical trials of HBOCs, suggesting further research is needed to better define indications and regimens of use (Buehler and Alayash, 2004). Intraoperative trials using trauma victims treated with red blood cell substitutes can only indirectly address the efficacy or safety of these solutions as pre-hospital resuscitative fluids. Multi-center pre-hospital trauma trials have just started for PolyHeme, and are planned for Hemopure.

PRE-HOSPITAL RESUSCITATION

The goal of resuscitation is to restore and maintain oxygen delivery to tissues. Hemorrhage reduces blood volume, and, sequentially, venous return, cardiac output and tissue perfusion. Oxygen delivery is reduced both by decreased blood flow and lowered hematocrit. A compensatory response to hemorrhage is an autotransfusion or transcapillary refill of interstitial fluid, which causes some hemodilution before resuscitation. Standard fluid resuscitation is to use asanguineous, clear crystalloids or colloids, which restore vascular volume and cardiac output. Oxygen delivery is increased as well but, owing to the hemodilution caused by transcapillary refill and volume infusion, the increase can be modest. The rationale for using an HBOC as a resuscitative fluid is straightforward: it is a solution that increases blood volume, blood flow AND oxygen content.

Despite a huge clinical need and a multi-billion dollar industrial investment in HBOC development, no product has yet received approval in either the US or Europe. Hemopure has been approved in South Africa, and a veterinary HBOC, Oxyglobin, has received FDA approval. In the following section we will review some of the unique properties of HBOCs, and their complicated record of preclinical and clinical efficacy and safety. We will also review evidence of how HBOCs expand plasma volume, the relationships between filling pressure and cardiac output, the direct cardiac effects of HBOCs, and the data on how oxygen uptake and delivery are affected by the use of HBOCs. Finally, we will discuss how HBOCs have been used in animal models of trauma, and as fluids for hypotensive resuscitation.

PLASMA VOLUME EXPANSION

All HBOCs have a colloid osmotic pressure at or above that of normal plasma (Table 12.2). Importantly, most surgical patients have COPs below normal as a result of a variety of pathologic and iatrogenic mechanisms. Trauma patients often have reduced COPs resulting from blood loss and transcapillary refill. Thus, all HBOCs have a COP higher than that of most trauma patients. Volume expansion with HBOCs should be significant, and is likely to be equal to or better than that achieved with conventional colloids. Fischer *et al.* (1999) compared plasma volume (PV) expansion (ΔPV) after a 30-minute infusion of 20 ml/kg 10 per cent DCLHb with a COP = 34 mmHg, with that found after an infusion of iso-oncotic 7.8 per cent human albumin or 60 ml/kg of LR in conscious sheep under conditions of normovolemia and hemorrhagic hypovolemia. The ΔPV for DCLHb calculated from Evans Blue indicator dilution and confirmed by hematocrit dilution was nearly two times greater than for albumin (Figure 12.1). The relatively increased expansion of 10 per cent DCLHb versus 7.8 per cent human albumin was unexpected, as the albumin was prepared to be an iso-oncotic control to the DCLHb. The explanation for the enhanced volume expansion of DCLHb is unknown, but several mechanisms can be hypothesized. PV enhancement could be the result of a reduction in capillary pressure due to arteriolar vasoconstriction. Alternatively, increased lymphatic pumping could return interstitial protein into the circulation and augment the plasma COP and expansion. Indeed, Fischer *et al.* (1999) did

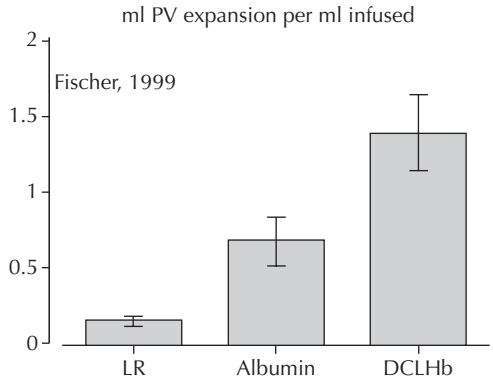

Figure 12.1 Volume expansion efficiency calculated as ml plasma volume (ΔPV) divided by ml infused. Data taken from Fisher *et al*. (1997) for a 30-minute infusion of 20 ml of DCLHb into hemorrhaged sheep. Control groups received a 30-minute infusion of 60 ml/kg of RL or 20 ml/kg of human albumin (Fischer *et al*., 1997).

report an increased plasma protein concentration, increased total vascular plasma protein and increased COP in the DCLHb group, despite the fact that the albumin and DCLHb were matched for volume infused and COP.

Oxyglobin is an FDA-approved veterinary HBOC made from bovine hemoglobin (Biopure) with a higher COP (~40 mmHg) than Biopure's human HBOC, Hemopure (26 mmHg). Oxyglobin was also found to be a potent volume expander, increasing blood volume more than Hespan in a study by Lurie *et al*. (2002). The hyperoncotic properties of Oxyglobin for veterinary treatment of anemia were demonstrated by Lurie *et al*. (2002), in which ΔPV was measured with Evans Blue dilution after infusing hetastarch and Oxyglobin in hypovolemic anesthetized rabbits. A 30-ml/kg infusion of Oxyglobin produced a ΔPV of 40 ml/kg, which was more than the 28 ml/kg expansion produced by an equal volume of Hextend. These investigators then performed a second infusion in the same animals using 30 ml/kg of Oxyglobin or Hextend in a crossover design; this time Hextend produced a larger ΔPV of 79 ml/kg, and a second infusion of Oxyglobin increased PV by an astounding 100 ml/kg. Lurie *et al*. (2002) concluded that the relative volume expansion of the initial infusion was much less than predicted by their measurements of the colloid oncotic pressure of Oxyglobin (40.4 mmHg) and Hextend (28.6 mmHg). On the other hand, the second infusion resulted in a very augmented volume expansion of Hextend and particularly of Oxyglobin.

There is a physical explanation for why a second dose of a colloid would augment volume expansion more than an equal first dose. The relationship between COP and colloid concentration is not linear, but rather increases exponentially due to macromolecular interactions (Hint, 1971; Till *et al*., 1989). Initial infusion of colloids produces low plasma concentrations and lowered volume expansion as compared with results that occur as infusion continues. The infusion of a second dose of colloids results in high plasma concentration, a larger macromolecular excluded space, and increased COPs. Lurie *et al*. (2002) used a large total dose of 60 ml/kg, and their data suggest that there can be a risk of hypervolemia and volume overload with such large doses of HBOCs, as with any colloid.

One key component to defining the utility and safety of any intravenous fluid is to define its volume expansion effects. Despite this, there are few data in the literature on the measured plasma volume expansion caused by Hemopure or PolyHeme – the two HBOCs now being proposed for pre-hospital use. No direct comparisons have been made with these products in animal trials, nor have there been any clinical evaluations of volume expansion with any HBOC.

CARDIAC OUTPUT

Asanguineous fluids expand vascular volume and increase cardiac output, but dilute red blood cells and oxygen content. Resuscitation with standard 'clear' IV fluids can increase cardiac output several fold, thereby effectively increasing oxygen delivery from depressed levels associated with shock to normal or even supranormal levels. Augmentation of supranormal levels of cardiac output with standard fluid resuscitation often occurs without full restoration of blood pressure, presumably because of lowered viscosity and widespread vasodilation from local autoregulatory mechanisms. However, many (if not all) HBOCs appear to impair cardiac output enough such that DO_2 is not increased above that reported for conventional volume expanders.

The goal of volume expansion is almost always to increase venous return and cardiac output. Reports of HBOC infusion have been shown to have no effect, a modest increase, or an actual decrease in cardiac output (Fischer *et al*., 1999; Krieter *et al*., 1997; Driessen *et al*., 2001). Cardiac output could be reduced by the increase

in left and right heart afterload known to occur as a result of vasoconstriction. Most evidence suggests that the systemic and pulmonary hypertension seen after HBOC infusion is the result of binding or scavenging of nitric oxide (NO) by interstitial hemoglobin. This blocks the normal basal level of vascular dilation because of nitric oxide diffusion from the endothelial cell to smooth muscle. It is hypothesized that polymerized HBOCs cause less nitric oxide binding and vasoconstriction than the tetramer HBOC because of reduced vascular leakage into the interstitium. Indeed, as the molecular size of HBOCs increases the vasoconstrictor response does decrease, but vasoconstriction is not eliminated (Sakurai *et al.*, 1997). While polymerized HBOCs may be comparatively less vasoconstrictive, recent studies of the polymerized HBOCs, including PolyHeme and Hemopure, have shown depressed cardiac output in swine (McNeil *et al.*, 2001; York *et al.*, 2003; Dubick *et al.*, 2004).

Figure 12.2 shows ΔCO plotted versus right atrial pressure for LR, albumin, and DCLHb as calculated from data gathered in our laboratory (Fischer *et al.*, 1999; Brauer *et al.*, 1999a). In these studies right atrial pressure was decreased by hemorrhage and then increased by infusion of RL, albumin or DCLHb. These data suggest an altered 'Starling cardiac function curve' (filling pressure cardiac output) with DCLHb as compared to either RL or albumin.

One hypothesis suggested for the apparent depression of cardiac output is that HBOCs do not increase cardiac output because HBOCs increase oxygen delivery. However, this explanation is not satisfactory, because all other volume expanders, including whole blood, increase oxygen delivery. Further, increased F_iO_2 alone does not reduce cardiac output.

Vane *et al.* (2002) reported depressed cardiac output and some deaths in animals treated with DCLHb after large-volume LR treatment of hemorrhage in an anesthetized model of a major abdominal surgical procedure. Vane *et al.* concluded that the combination of vasoconstriction, hypervolemia and cardiac depression likely contributed to the poor outcomes. Depressed cardiac output has been reported in several clinical trials of both tetramer HBOCs and polymerized HBOCs (Hughes *et al.*, 1995; Standl *et al.*, 1997; Kasper *et al.*, 1998; Garrioch *et al.*, 1999; Levy *et al.*, 2002). These data suggest that some level of cardiac dysfunction or impairment can occur with some, if not at all, HBOCs. Some effort should be

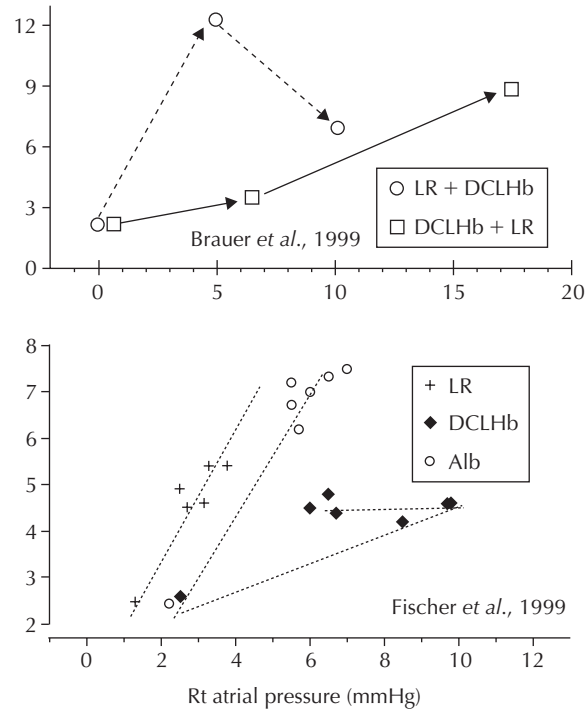

Figure 12.2 Cardiac output in l/min (y-axis) is compared with filling pressure in hemorrhaged sheep both before and after infusion with RL (60 ml/kg), Albumin (20 ml/kg) and DCLHb (20 ml/kg) (Fischer *et al.*, 1997). Brauer *et al.* (1999b) infused LR (60 ml/kg) both before and after DCLHb (20 ml/kg) in hemorrhaged sheep.

generated to gather human volunteer and patient data comparing how infusion of HBOCs and traditional plasma expanders alter cardiac output, right atrial pressure and blood volume. Additionally, evaluation of cardiac function using ultrasound analysis could provide valuable data on ventricular wall motion and contractility.

The mechanism for depression of cardiac output is not known. It may be caused by increases in left and right heart afterload. In general, the pulmonary circulation appears to be more sensitive to HBOCs, and pulmonary hypertension is a common finding after infusion of HBOCs – at least in laboratory animals. Unfortunately, the two most common laboratory animals for evaluating hemodynamics (swine and sheep) may have more potent vasoconstrictor responses than humans, and thus they may exhibit exaggerated vasoconstriction and hypertension. Alternatively, HBOCs may directly affect cardiac function. Muir *et al.* (2000) evaluated cardiac function in dogs during a 90-minute infusion of

30-ml/kg Oxyglobin. They documented a 20 per cent decrease in cardiac output and a 25-mmHg increase in afterload (mean arterial pressure). End-diastolic pressure doubled from 10 to 25 mmHg, and was associated with an increase in left ventricular end-diastolic volume and a reduction in ejection fraction. Further, dP/dt, a load-dependent index of contractility, was significantly reduced. Load-independent variables, such as the end-systolic slope, were not significantly changed. Importantly, neither was cardiac oxygen consumption. However, such changes do suggest caution when using HBOCs in the presence of any pre-existing ventricular dysfunction. More research on the direct cardiac effects of HBOCs is needed.

OXYGEN UPTAKE VERSUS OXYGEN DELIVERY

While evaluation of oxygen delivery has been a reasonable endpoint for judging all conventional resuscitative fluids, it may not be the best measure of HBOC effectiveness. There is a substantial amount of data showing that HBOCs can facilitate and enhance oxygen unloading from both plasma HBOC hemoglobin and from RBC hemoglobin (Intaglietta, 1999; Wettstein *et al.*, 2003). The even distribution of hemoglobin in the plasma may act as a 'stepping stone' to facilitate oxygen diffusion from red blood cell Hb to the endothelial wall (Winslow, 2000; McCarthy *et al.*, 2001; Tsai and Intaglietta, 2002). Additionally, HBOCs often have a *P*50 that is higher than that of blood, resulting in enhanced unloading. Reduced partial pressure of oxygen (PvO_2) in mixed venous blood and increased tissue oxygen levels with HBOC treatment have been reported and may reflect these mechanisms. This opposes the conventional explanation for reduced PvO_2, which is either reduced oxygen delivery or increased tissue metabolism. A new paradigm may be needed for both treatment and monitoring of HBOC use. Several studies do show better tissue oxygenation and improved lactate levels with HBOC resuscitation (Pickelmann *et al.*, 1996; Nolte *et al.*, 1997; McNeil *et al.*, 2001; Wettstein *et al.*, 2003). Such encouraging results provide evidence that HBOCs may have a role in resuscitation of hemorrhage. Unfortunately, such changes have not always been observed, and further data on how effective the enhanced oxygen unloading can be are needed before the full value of HBOCs as resuscitative fluids can be defined.

HYPOTENSIVE RESUSCITATION

A series of experiments sponsored by the US Air Force evaluated Hemopure as a small-volume formulation for hypotensive resuscitation in anesthetized swine (McNeil *et al.*, 2001; Sampson *et al.*, 2003; York *et al.*, 2003). When infused to a hypotensive target mean arterial pressure (60 mmHg), the volume sparing of an HBOC can be profound because of the high COP and induced vasoconstriction. Outcomes measured in an anesthetized 60-kg swine model were equal or better for a small-volume infusion of Hemopure as compared with a large-volume infusion of RL or hypertonic saline dextran (HSD) (York *et al.*, 2003). However, acute doses of RL and HSD studied were exceedingly high (19+ l of LR and 1500 ml of HSD; Sampson *et al.*, 2003). The control animals may have been grossly over-resuscitated with both RL and HSD. A 1500-ml or 25-ml/kg dose of HSD is six times the recommended safe dose of 4 ml/kg. Figure 12.3(a) compares the volume requirements of Hemopure when used for hypotensive resuscitation to mean arterial pressure of 60 mmHg. Results are expressed as volume-infused per hemorrhaged volume for PolyHeme and Hemopure and the two FDA-approved fluids used for combat casualty care – RL and hetastarch. PolyHeme and Hemopure did cause notable reductions in cardiac output and venous oxygenation. Despite this, lactates were not elevated and survival was 100 per cent, as it was with larger volumes of conventional fluids. This suggests the interpretation that enhanced HBOC oxygen unloading occurred and that reduced venous oxygenation was evidence of enhanced tissue delivery of oxygen.

Recently, PolyHeme was provided to the US Army for independent animal testing using hypotensive resuscitation models in three independent laboratories. PolyHeme increased arterial oxygen content but did not increase oxygen delivery more than Hextend in hemorrhaged swine subjected to hypotensive resuscitation to systolic target pressures = 80 mmHg. PolyHeme depressed cardiac output when compared with hetastarch and RL (Dubick *et al.*, 2004). Neither did PolyHeme improve mortality versus Hextend in swine and rats (Sondeen *et al.*, 2004; Handrigan *et al.*, in press). It may be that small-volume or

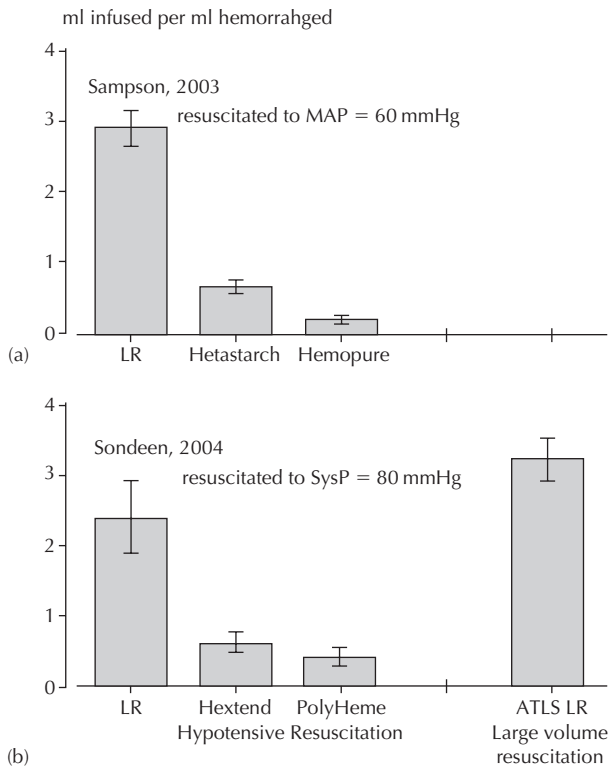

Figure 12.3 Relative volume requirements for two HBOCs (PolyHeme and Hemopure) compared to the FDA-approved fluids RL and Hextend, when each was infused until a hypotensive target pressure was achieved in hemorrhaged swine (Sampson *et al.*, 2003; Sondeen *et al.*, 2004).

hypotensive resuscitation with HBOCs is ineffective because of the limited dose. Resuscitation to hypotensive targets can reduce HBOC volume needs as compared with Hextend because of HBOC vasoconstrictor activity, but there was no apparent advantage in survival or physiology when compared with Hextend formulations. Volume sparing at equal efficacy is sufficient justification for using a safe HBOC for combat casualty care. Figure 12.3(b) shows volume requirements of PolyHeme versus RL and Hextend when used for hypotensive resuscitation. Survival of the hypotensive groups was only 33 per cent to 50 per cent as compared with 100 per cent survival with large-volume standard of care using RL. It is worth noting that hypotensive resuscitation with LR resulted in only slightly reduced volumes compared with full standard of care treatment.

HBOCs have rarely been evaluated in animal models of trauma; rather, hemorrhagic shock is used as a surrogate model of trauma. Exceptions to this approach are the studies using anesthetized swine combined with a modest hemorrhage with head injury (Gibson *et al.*, 2002) or blunt chest injury (Maxwell *et al.*, 2000). These studies compared several hemoglobin formulations, demonstrating increased mortality with a hemoglobin tetramer. Using the same model, DCLHb increased mortality, a result similar to the Baxter US trauma trial (Gibson *et al.*, 2002). Polymerized hemoglobin caused similar pressure responses to the tetramer but reduced mortality (Gibson *et al.*, 2002). Finally, Gibson *et al.* (2002) reported the best results with Baxter's recombinant hemoglobin designed so as not to bind nitric oxide. However, compared with RL, even this specially modified HBOC caused some elevation in pulmonary pressure and early depression of cardiac output (Malhotra *et al.*, 2003). In 2003 Baxter discontinued the development of all HBOC products, and as of 2004 all the major pharmaceutical companies have abandoned HBOC development.

NEW FORMULATIONS

Conjugated hemoglobins

Another approach to HBOC development has been the design of surface-decorated or conjugated hemoglobins, such as pyridoxylated hemoglobin polyoxyethylene (PHP) or polyethylene glycol-modified human hemoglobin (Hemospan®). Such modifications increase viscosity and COP. Hemospan (see Chapter 40) is a viscous, hyperoncotic formulation, despite having a dilute hemoglobin concentration of less than 5 g/dl. Furthermore, Hemospan has a low *P*50 of 5.5 mmHg (Kramer, 2003). Animal studies of Hemospan suggest that the free Hb in plasma unloads oxygen more efficiently as compared with red blood cell Hb because of the removal of the microcirculatory spatial heterogeneity imposed by cellular Hb (Winslow, 2000; McCarthy *et al.*, 2001; Tsai and Intaglietta, 2002). The unique formulation for Hemospan is based on several novel hypotheses. Only a small amount of Hb may be needed to facilitate oxygen unloading of red blood cells (Hartman *et al.*, 1998). In theory, the elevated oxygen affinity (low *P*50) of Hemospan delays early release and prevents vasoconstriction. Further, vasodilation may be induced by Hemospan's high viscosity, thus increasing blood endothelial shear forces and enhancing nitric oxide release (Tsai *et al.*, 1998).

In a recent animal trial of Hemospan versus pentastarch, vasoconstriction was evident as both pulmonary and systemic pressures were elevated using Hemospan as compared with pentastarch, but cardiac output was not altered (Drobin *et al.*, 2004). In another swine hemorrhage model, effective resuscitation with improved cardiac output was reported for PHP-Hb (Wirk and Vaslef, 2004). The concept that an HBOC with high oncotic pressures and high viscosity has enhanced efficacy is intriguing, and deserves further evaluation in clinically relevant models.

Hypertonic saline–HBOC combinations

Another approach to developing a small-volume resuscitative fluid could be to combine the vasoconstrictive HBOC with a vasodilator fluid or drug. One possible formulation would be a combination of hypertonic 7.5 per cent saline plus an HBOC (HSHb). Such mixtures have been suggested as an improvement to increasing oxygen delivery by replacing dextran with an HBOC and assuming cardiac output is increased equally. However, an analysis of experimental data suggests that hypertonic saline dextran is almost as effective as HSHb, assuming equivalent cardiac output augmentation (Kramer *et al.*, 1997; Poli de Figueiredo *et al.*, 2001). The small difference in oxygen delivery results from the small amount of Hb used in a small-volume formulation. Polymerized bovine hemoglobin (PolyBvHb) combined with 7.5% NaCl was more effective in improving blood pressure when infused into hemorrhaged dogs, and also improved cardiac output and mesenteric blood flow more than either HBOC or hypertonic saline alone. However, no comparisons were made against other hypertonic saline–colloid formulations. Nevertheless, these studies are promising and suggest further evaluation of the concept of a hyperosmotic HBOC.

The combination of $\alpha\alpha$-Hb with a vasodilatory hypertonic acetate component was suggested with the rationale that the vasodilatory acetate would offset the cardiac depression and vasoconstrictor properties of hemoglobin (Poli de Figueiredo *et al.*, 1997). The solution was tested in hemorrhaged swine and did produce improved cardiac output, but it also caused severe cardiovascular instability. Pulmonary hypertension coupled with systemic hypotension was severe in some animals and led to cardiovascular collapse.

SUMMARY

The key beneficial mechanism by which HBOCs may be uniquely effective as resuscitative solutions is their ability to enhance oxygen unloading. Animal studies often demonstrate reduced lactates and improved tissue oxygen. On the other hand, vasoconstriction and depressed cardiac output may limit the utility of HBOCs. Polymerization and new formulations may reduce the vasoconstrictive effects compared with tetramer HBOCs, but they do not appear to be eliminated by any formulation. We await results from ongoing and planned trauma trials using polymerized HBOCs, and further testing of new formulations. If HBOCs are shown to be safe and effective in civilian trauma trials, they may indeed be the ultimate solution for combat casualty care.

EDITOR'S SUMMARY

Trauma, on the streets, on the battlefield, or in the surgical suite, represents one of the prime targets for blood substitutes. In this application, time is critical; immediate stabilization of a trauma victim in the first hour after the insult (the 'golden hour') has become associated with the maximal survival rate. Valuable time can be lost in cross-matching blood but, of perhaps more importance, it is likely that some acellular oxygen carrying resuscitation fluid is possibly the best solution to use in the golden hour. Traumatologists and military medical personnel have been frustrated at the slow pace of development of blood substitutes, and some of the reasons for this are discussed.

Research in the past decade has indicated that the optimal resuscitation protocol may be one in which the mean arterial pressure is not fully restored immediately. When volume resuscitation is too aggressive, rebleeding can be a consequence. Such continuation of hemorrhage after initial control can be especially harmful, leading to excessive blood requirements, coagulopathies and multiorgan failure. Thus a better protocol may be to achieve enough restoration of pressure so that tissue perfusion is reestablished

in order to prevent tissue damage and to allow sufficient time for surgical control of hemorrhage.

One problem has been that there is no good animal model for real trauma, which combines blood loss with often both penetrating and blunt injury. Intraoperative models or controlled hemorrhage are probably too simple to serve as useful models to evaluate efficacy and long-term survival after treatment. A second problem is that there is no good agreement on what the properties of the ideal resuscitation solution should be. One of the often overlooked properties is oncotic pressure – the ability of the infused solution to increase and maintain the vascular volume. Some of the underlying physical chemistry relating to oncotic pressure is still not completely understood, and why some of the solutions seem to be more oncotically active than others is also not clear. As a general class, hemoglobin solutions tend to depress cardiac output in spite of their oncotic activity, and this effect needs to be understood before safe solutions can be approved for use. Finally, a unique requirement for the military is minimization of the volume needed for resuscitation, since logistic constraints often govern how much fluid is available on the battlefield.

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Chapter 13

Surgical Hemorrhage

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INTRODUCTION

The development of blood substitutes spans the past 75 years, but progress in the last decade has been meteoric (see Chapter 1). The current generation of blood substitutes employed in clinical trials are fundamentally red blood cell (RBC) substitutes – i.e., they are designed primarily to transport oxygen. The products that are now being tested in US Food and Drug Administration (FDA) phase III clinical trials are derived from hemoglobin and are thus often referred to as hemoglobin-based oxygen carriers (HBOCs). The objectives of this chapter are to outline potential clinical applications of HBOCs for acute blood loss and discuss recent patient trials.

POTENTIAL CLINICAL BENEFITS OF HBOCs IN SURGICAL CARE

The potential benefits of HBOCs in surgical care can be categorized into the areas of availability, safety and efficacy (Table 13.1). Perhaps most conspicuous in the management of the patient with acute blood loss is the scenario when stored blood is not available. The logistic problems of access to typed cross-matched packed red blood cells (RBCs) for civilian trauma and the compelling need for an HBOC in military trauma is outlined in Chapter 12. There are patients whose demise is attributable to a delay or lack of blood availability in the US today despite sophisticated trauma systems, and the demand for an HBOC in the current war against global terrorism is clear.

Stored blood is reportedly safer than ever due to comprehensive screening for disease transmission (see Chapter 2), but the potential adverse effects of packed RBC storage on the immune response to injury and illness are becoming more apparent (Silliman *et al.*, 2004). We have been interested in the proinflammatory effects of stored RBCs and, specifically, their capacity to provoke neutrophil polymorphonuclear (PMN) cytotoxicity.

The PMN is a key cellular mediator in the pathogenesis of post-injury multiple organ failure (MOF). Consequently, PMN functional responses are evaluated as a clinical surrogate for the two-event model of MOF – i.e., inflammatory

Table 13.1 Potential clinical benefit of hemoglobin-based oxygen carriers in surgical care

Availability	Abundant supply
	Universally compatible
	Prolonged shelf-life
	Storage at room temperature
Safety	No disease transmissions
	No antigenic reactions
	No immunologic effects
Efficacy	Enhanced oxygen delivery
	Improved rheologic properties

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priming and subsequent activation (Botha *et al.*, 1995). The two-event construct of post-injury MOF is based on the fundamental concept that injury primes the innate immune system such that a second insult, during this vulnerable window, provokes unbridled systemic inflammation resulting in organ dysfunction. PMN priming/activation is typically studied as the *in vitro* surrogate of the two-event phenomenon. Priming is defined as an enhanced response to a stimulus that is due to prior exposure of the cell to a different agonist (Ingraham *et al.*, 1982). In our ongoing epidemiologic studies (Sauaia *et al.*, 1994), we have shown that more than six units of RBC transfusion within the first 12 hours post-injury is an independent risk factor for MOF (Moore *et al.*, 1997). Furthermore, the age of transfused blood within the first 6 hours post-injury correlates with the incidence of MOF (Zallen *et al.*, 1999). Previous studies in our center have shown that after severe injury, patients at high risk for MOF have circulating PMNs that are primed for cytotoxicity within the first 6 hours post-injury, as marked by the increased surface expression of CD11b/CD18, p 38 MAPK activation, release of cytotoxic products in response to fMLP, and delayed apoptosis (Biffl *et al.*, 1999).

The precise mechanism(s) linking packed RBC transfusion and PMN priming remains to be established, but it is generally believed that passenger leukocytes accompanying RBCs in storage are important in the generation of pro-inflammatory agents (Bordin *et al.*, 1994). Plasma from stored RBCs primes PMNs *in vitro*, and this effect has been shown to increase progressively from 14 to 42 days of storage (Nielsen *et al.*, 1996). Some investigators (Shanwell *et al.*, 1997) have incriminated cytokines (TNF-α, IL_1, IL_6, IL_8) generated during storage while we have focused on proinflammatory lipids presumably generated from the RBC membrane (Silliman, 1997).

Metabolites of the arachidonic acid cascade have been strongly implicated in the pathogenesis of transfusion-related acute lung injury (Silliman *et al.*, 1998). Although prestorage leukoreduction of RBCs decreases the generation of cytokines (Shanwell *et al.*, 1997), this process does not eliminate PMN priming (Biffl *et al.*, 2001). The administration of a blood substitute, in lieu of stored packed RBCs, may avoid these immunomodulatory consequences in high-risk patients (Moore, 2003).

Finally, efficacy for the current generation of HBOCs is focused on enhanced oxygen delivery to tissue. Packed RBCs undergo shape change within 2 weeks of storage and this progresses during longer preservation (Berezina *et al.*, 2002). The effects of reduced RBC deformability on microcirculation have been documented experimentally, and include impaired tissue access of the stiffened RBC and RBC entrapment resulting in microvascular obstruction (Doyle *et al.*, 1990). These experimental findings might explain the observation that blood transfusion fails to improve oxygen consumption in critically ill and injured patients (Marik and Sibold, 1993). Moreover, due to their rheologic advantages, HBOCs may improve oxygen delivery to tissue with mechanical vascular inflow obstruction – e.g. reperfusion edema or atherosclerotic disease (see Chapter 17).

POTENTIAL ROLE OF HBOCs IN SURGICAL CARE

Table 13.2 lists some potential applications of HBOCs in surgical care.

Table 13.2 Potential role of hemoglobin-based oxygen carriers in surgical care

Application	Location
I. Perioperative applications	
Reduce allogeneic RBC transfusions	ED, angiography, OR, ICU
Attenuate transfusion immunomodulation	OR, ICU
II. Acute hemorrhagic shock	
When stored RBCs unavailable	Field, ED, OR, ICU, remote hospital, civilian disaster, military conflict
More efficient resuscitation	Field, ED, OR, ICU
Low-volume resuscitation	Remote hospital, civilian disaster, military conflict
III. Regional perfusion	
Enhance oxygen delivery	
Ischemic reperfused tissue/organ	OR, ICU
Inflamed tissue	OR, ICU
Tumor radiosensitivity	Hospital
Ex vivo organ perfusion	Hospital, OR

ED = emergency department; OR = operating room; ICU = intensive care unit.

FDA approval of a new product proceeds through phase I, II, and III studies designed to establish safety and efficacy (see Chapter 3). FDA regulation defines efficacy as follows:

> Effectiveness means a reasonable expectation that ... pharmacologic or other effects of the biologic product ... will serve a chemically significant function in the diagnosis, cure, mitigation, treatment or prevention of disease in man.

The Center for Biologics Evaluation and Research (CBER) is the review body for the FDA in the arena of biologies, and has published a comprehensive listing of 'points to consider in the safety evaluation of HBOCs' (CBER, 1991).

These points encompass characterization of the product, animal safety testing, and human studies; and address the theoretic concerns regarding Hb solutions (i.e., vasoconstriction, organ dysfunction, oxidative tissue injury, synergy with bacterial pathogens, and immunomodulation). In 1994, the CBER convened a workshop with the National Heart, Lung and Blood Institute and the Department of the Army to develop 'points to consider in the efficacy evaluation of HBOCs' (CBER, 1994). Clinical trial endpoints were divided into two categories. Direct measures of clinical benefit included improved patient survival and reduced complications; an example of a surrogate endpoint was a laboratory measurement expected to correlate meaningfully with clinical benefit. Documenting a direct clinical endpoint for HBOCs was viewed as challenging, because this endpoint had never been established for RBCs. Specific recommendations for clinical studies were in three areas: perioperative applications, acute hemorrhagic shock, and regional perfusion.

Field trials for trauma where packed RBCs are not available were admonished as challenging because of safety and ethical issues. Decreased perioperative allogeneic RBC transfusion was also recognized as a significant benefit, but the potential risks of HBOCs would have to be defined and contrasted to packed RBCs. Suggested regional perfusion studies with HBOCs included enhanced tumor radiosensitivity and an adjunct during coronary angioplasty (the FDA had approved Fluosol DA in 1989 as an oxygen-carrying drug for this setting).

EXCEPTION FROM INFORMED CONSENT FOR TRAUMA TRIALS

Clinical investigation of HBOCs for emergent resuscitation of post-injury hemorrhagic shock is challenging due to the unpredictable, often life-threatening situation and the time constraints for effective intervention. By definition, critically injured patients are unable to provide informed consent and, due to the exigent circumstance, legal guardians or next of kin are often not accessible or appropriate for proxy consent during the narrow therapeutic window. One of the recent major advances in trauma research is the FDA codification of 'Exception from Informed Consent Requirements for Emergency Research', detailed in the Code of Federal Regulation, Title 21, part 50, section 24 (21CFR 50.24), which became effective 1 November 1996 (DHHS/FDA, 1996). The seven fundamental components of this regulation are outlined in Table 13.3. Research protocols using this Exception from Informed Consent must be conducted under a separate investigational new drug (IND) application to the FDA. Studies may proceed only after a sponsor has received written permission from the FDA and the Institutional Review Board (IRB) has documented that the requirements have been met.

We have employed and continue to employ 21CFR 50.24 for the clinical investigation of

Table 13.3 Exception from informed consent requirements for emergency research (21CFR 50.26)

1. Human subjects are in life-threatening situation; available treatments are unsatisfactory.
2. Obtaining informed consent is not feasible.
3. Participation in the research holds out the prospect of direct benefit to the subjects.
4. The clinical investigation could not practicably be carried out without the waiver.
5. The investigational plan defines the length of the potential therapeutic window; investigator has committed to attempting to contact a legally authorized representative during that window.
6. The IRB has approved the informed consent document and procedures.
7. Additional protection of the rights and welfare of the subjects include: community consultation, public disclosure, establishment of an independent data monitoring committee, and consent to continue the study is obtained from the patient as soon as possible.

HBOCs in the initial resuscitation of critically injured patients manifesting evidence of hemorrhagic shock. Our experience with these regulations is similar to others, but several issues specific to trauma warrant emphasis. Meeting the first four criteria in the Requirements (Table 13.3) is relatively straightforward. Defining the therapeutic window on a scientific basis, however, can be difficult, and detailing the process for attempting to contact a legally authorized representative for written informed consent to continue the study during this window is imperative.

Finally, satisfying Community Consultation and Public Disclosure can be time-consuming. Fortunately, trauma registries are helpful in defining the potential catchment area for injured patients likely to be enrolled in a pre-hospital resuscitation trial. Community Consultation refers to ensuring that communities in which the study will transpire and from which the study patients will be accrued are involved in the IRB decision-making for approval. At times, the destination between soliciting community opinion versus seeking approval can be blurred. Public Disclosure refers to informing the public, communities and researchers about the study purpose, scientific rationale, patient risks and potential benefits, and that informed consent will not be obtained at the time of enrollment. This process must be done (1) before the trial is initiated and (2) after the research is completed. The FDA expects that multiple forums and media resources will be employed for wide dissemination of this information. Competing for time or space within conventional news media, however, can be frustrating due to unpredictable delays, and occasionally reporters misinterpret the information.

Ultimately, achieving compliance with the Requirements demands a committed and cohesive research team. There are a number of valuable adjunctive members, but we found the essential core for our pre-hospital HBOC studies included (1) public relations staff, (2) study coordinator, (3) chief paramedic, (4) paramedic physician advisor, (5) director of trauma services, and (6) principal investigator. The public relations member was pivotal in establishing contacts with the relevant news media (newspapers, television, radio), arranging public meetings and maintaining a dialogue with key community representatives. The study coordinator role was full time; developing information for the institutional website, preparing information packets and slide presentations, distributing questionnaires for public meeting and maintaining communication with the IRB and sponsor. The trauma service director, paramedic advisor and chief paramedic each participated in the formal presentations, ranging from 30 to 60 minutes in duration. All members of the core team attended the multiple evening and weekend meetings with the participating communities. Despite the added work and frustrations associated with achieving FDA and IRB approval for Exception to Informed Consent, it is a valuable aspect of planning and conducting emergent resuscitation research.

CLINICAL EVALUATION OF TETRAMERIC HEMOGLOBIN IN TRAUMA

Of the modified Hb tetrameric solutions that looked promising in the late 1980s, only one formulation was authorized by the FDA for a phase III study in trauma. HemAssist (Baxter Healthcare, Boulder, CO) consisted of Hb tetramers crosslinked between alpha subunits with DBBF to prevent dissociation into dimers and reduce oxygen affinity. Unfortunately, this product failed (see Chapters 34 and 35). Regarded by some as a major setback for the clinical implementation of HBOCs, it is important to emphasize that this US multicenter trial of diaspirin crosslinked Hb (DCLHb) for the treatment of severe traumatic hemorrhagic shock was based on the explicit proposal that 'DCLHb was tested not as a substitute for blood but rather as an adjunct to the currently used therapies for enhancing oxygen delivery: fluids, blood, and operative intervention' (Sloan *et al.*, 1999). The authors rationalized this study design because in preclinical trials 'DCLHb has been shown to be effective in enhancing perfusion in small volumes, suggesting a pharmacologic effect that is independent of hemoglobin'. However, the pharmacologic effect was not always reported as beneficial.

Investigators at the Letterman Army Institute of Research (Hess *et al.*, 1993) reported that, in a swine model of hemorrhagic shock, DCLHb infusion doubled systemic and pulmonary vascular resistance and these responses were associated with a fall in cardiac output. In fact, these changes were equivalent to resuscitation with unmodified tetrameric Hb. The authors concluded, 'The decrease in cardiac output associated with the vasoconstriction in the Hb-treated animals was

equal to the increase in oxygen-carrying capacity … crystalloid or colloid solutions provided equally rapid correction of the elevated whole blood lactate'. In a follow-up study (Poli de Figueiredo *et al.*, 1997), the infusion of low-dose (4 ml/kg = 14 g Hb) DCLHb into swine subjected to hemorrhagic shock prompted the authors to further warn that 'pulmonary hypertension and low peripheral perfusion may offset benefits for trauma patients'. Although the authors of the DCLHb trial cited several animal models that appeared to support their study hypothesis, none of these models replicated their study design – a lesson for future conduct of clinical trials with HBOCs.

CLINICAL SAFETY OF POLYHEME™ IN TRAUMA

At this moment, the most successful HBOCs in clinical trials have been polymerized Hb solutions. Perhaps a coincidence, but polymerization addresses several of the problems inherent in tetrameric Hb – i.e., enhanced intravascular retention and reduced colloid osmotic activity (see Chapter 30). Polymerization also appears to attenuate vasoconstriction associated with the infusion of Hb solutions. A proposed explanation is that tetrameric Hb (65 kDa) extravasates through the endothelium to bind albuminal nitric oxide, leading to unopposed vasoconstriction; but polymerized Hb ($\geqslant$ 130 kDa) remains in the vasculature to bind only luminal nitric oxide (Gould and Moss, 1996). Of interest, Hb of the common earthworm, *Lumbricus terrestris*, is a polymer with a molecular weight of 400 kDa that circulates extracellularly (Fushitan *et al.*, 1986). Mice and rats undergoing exchange transfusion with this naturally occurring polymeric Hb showed no changes in behavior, and nuclear magnetic resonance spectroscopy of the heart confirmed normal oxygen-carrying capacity (Hirsh *et al.*, 1997).

Polymerized HBOCs have undergone extensive preclinical and clinical testing for safety. Hemopure (Biopure Corp, Cambridge, MA), a polymer of bovine Hb, has been used successfully to reduce allogeneic RBC transfusion in elective cardiac, aortic and hepatic surgery (see Chapters 36 and 37). One study with abdominal aortic reconstruction raised concern about increased systemic vascular resistance (Kasper *et al.*, 1996), an effect identified in normal volunteers. Animal studies designed to replicate pre-hospital hypotensive resuscitation for hemorrhagic shock in the military setting have also been encouraging. Hemopure has been approved for replacement of acute blood loss in South Africa, but there are no published results to date.

Clinical testing of Hemolink (Hemosol, Inc., Mississauga, Ontario, Canada), o-raffinose polymerized Hb, has targeted conservation of allogeneic red blood cell transfusion through enhanced intraoperative autologous donation during coronary artery bypass grafting. The pulmonary and systemic vasoconstriction associated with Hemolink infusions are attenuated by general anesthesia (Ning *et al.*, 2000). Assessment of Hemolink for hemorrhagic shock and regional reperfusion has been limited. The mechanism(s) responsible for vasoconstriction with some of the polymerized HBOCs has not been established, but is speculated to be largely due to residual tetrameric Hb binding to nitric oxide. Alternative mechanisms have been proposed, including increased endothelin release, enhanced adrenergic receptor sensitivity, and reduced arterial wall shear stress (see Chapter 8).

PolyHeme (Northfield lab, Evanston, IL) has been the only polymerized HBOC to be evaluated in trauma patients in the US to date (see Chapter 30). Under FDA guidance, we initiated clinical trials in trauma to confirm safety with escalating doses of PolyHeme. In the first clinical trial, 39 patients received 1 (n = 14), 2 (n = 2), 3 (n = 15) or 6 (n = 8) units of PolyHeme instead of stored red blood cells as part of their initial resuscitation after acute blood loss (Gould *et al.*, 1997). Infusion rates ranged from 1 unit in 175 minutes to 6 units (300 g) in 20 minutes. Although the RBC Hb fell to 2.9 $\pm$ 0.2 g per cent, total Hb was maintained at 7.5 $\pm$ 0.2 g per cent with PolyHeme. With respect to safety, the patient's temperature, mean arterial pressure, heart rate and creatinine clearance did not change during the 72-hour study period. Liver function tests and amylase varied substantially because of the patient's injuries.

Cognizant of the vasoconstriction associated with the DCLHb clinical trial, we designed a study specifically to evaluate the vascular response to PolyHeme infusion in acutely injured patients (Johnson *et al.*, 1998). Patients requiring urgent transfusion were randomized to either PolyHeme (up to 6 units) or stored red blood cells during their initial resuscitation. Systemic arterial pressure, pulmonary arterial pressure, cardiac index

and pulmonary capillary wedge pressure were measured every 4 hours post-infusion for 24 hours. There were no significant differences between the groups for these indices, or the calculated systemic or pulmonary vascular resistance.

Additional potential problems reported with the clinical use of polymerized Hb solutions include interference of laboratory tests that are based on colorimetric changes from dissolved plasma Hb, inaccuracy of oxygen saturation monitoring because of methemoglobin, mild elevations of serum amylase (but without evidence of pancreatitis) and skin rashes. None of these issues have been considered clinically important adverse events to date.

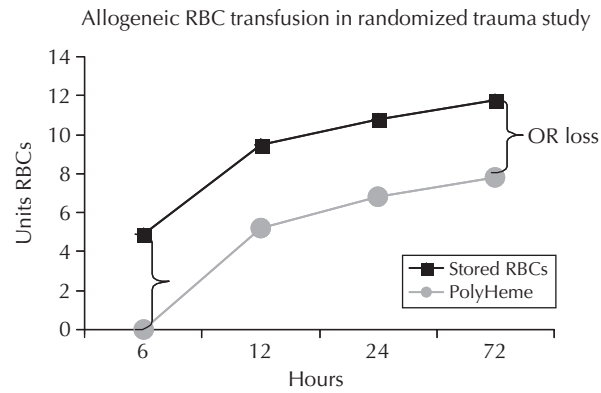

Figure 13.1 Acutely injured patients were randomized to receive stored RBCs or up to 6 units of PolyHeme as their initial blood replacement. The net difference in allogeneic RBC transfusion persisted for 72 hours, and was due to the initial operating room loss. * $P < 0.05$. (From Moore, 2003, with permission.)

CLINICAL EFFICACY OF POLYHEME TRAUMA CARE

Reduction of allogeneic RBC transfusions

Prompted by the FDA guidelines to demonstrate efficacy, all HBOC companies have pursued what appeared to be the simplest clinically – i.e., to reduce the need for allogeneic RBC transfusions. In collaboration with David B. Hoyt, MD, and the University of California at San Diego, we conducted a randomized trial in patients requiring urgent transfusion (Gould *et al.*, 1998). The 44 trauma patients (Injury Severity Score [ISS] = 21 ± 1.3) were allocated to receive stored RBCs or up to 6 units of PolyHeme as their initial blood replacement. The RBC Hb was equivalent pre-infusion (10.4 ± 0.4 g/dl versus 9.4 ± 0.3 g/dl); at end infusion, the RBC Hb of the PolyHeme patients fell to 5.8 ± 0.5 g/dl versus 10.6 ± 0.3 g/dl in the control. The PolyHeme group received 4.4 ± 0.3 units, resulting in a plasma Hb of 3.9 ± 0.2 g/dl. The total number of allogeneic RBC transfusions for the control versus PolyHeme was 10.4 ± 0.9 units versus 6.8 ± 0.9 units ($P < 0.05$), respectively, through day 1, and 11.3 ± 0.9 units versus 7.8 ± 0.9 units ($P = 0.06$), respectively, through day 3. After the initial phase, infusion of 4.6 units of stored RBCs in the control group was equivalent to the 5.2 units in the PolyHeme group. Both volumes presumably represented the infused RBCs or PolyHeme lost during acute hemorrhage. Subsequent replacement volumes were comparable, ultimately sparing the PolyHeme group approximately 4 units of allogeneic RBC transfusion (Figure 13.1).

With our long-term interest in the pathogenesis of post-injury MOF, we then pursued the hypothesis that PolyHeme, in lieu of stored RBCs during initial resuscitation, would attenuate transfusion-associated immunomodulation (TRIM) of allogeneic RBC transfusion.

In preparation for these clinical trials, we conducted *in vitro* studies to test our hypothesis that PolyHeme – free of inflammatory cytokines and lipids – would eliminate the PMN priming previously documented with stored RBCs (Aiboshi *et al.*, 2001). Human PMNs were isolated from healthy volunteers and the plasma fraction was separated from packed RBCs at 42 days of storage in our blood bank (the last day stored RBCs can be transfused clinically, but are often the first RBCs infused into trauma patients). The isolated PMNs were incubated with either RBC plasma or PolyHeme at concentrations calculated to be equivalent to 8 units of transfusion. The plasma fraction representing 3 or more units of stored RBCs primed the human PMNs for enhanced superoxide production and elastase release (Figure 13.2).

In our subsequent clinical trial, injured patients requiring urgent transfusion were administered either PolyHeme (up to 20 units = 1000 g) or stored RBCs for their initial resuscitation (Johnson *et al.*, 2001). PMN priming was determined by the surface expression of CD11b/CD18 in whole blood and superoxide production in isolated

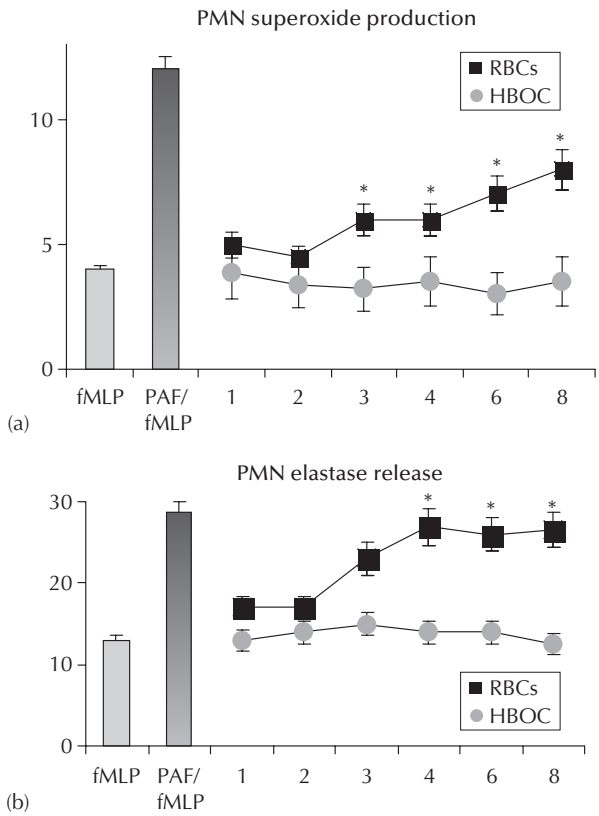

Figure 13.2 Isolated human neutrophils (PMNs) were incubated with either the plasma fraction from stored RBCs or PolyHeme at concentrations equivalent to 1 through 8 units of acute transfusion. (a) PMN superoxide production;(b) PMN elastase release. FMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet activating factor. * $P < 0.05$. (From Moore, 2003, with permission.)

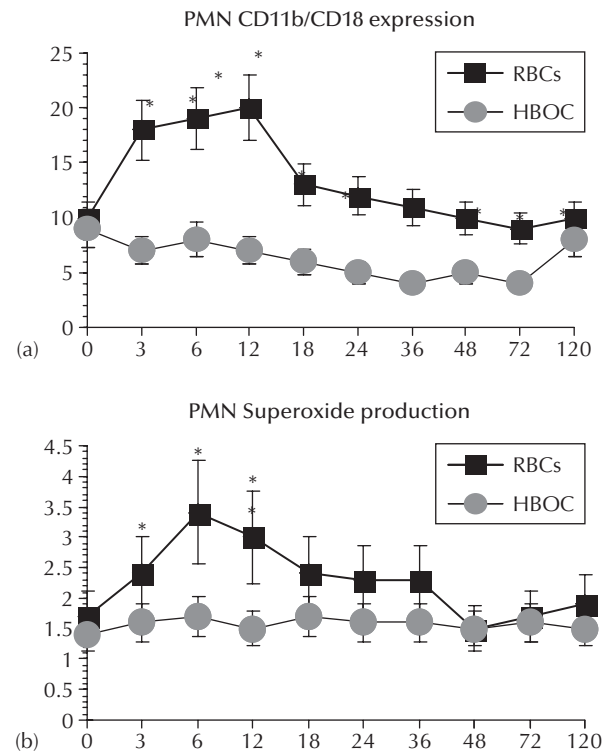

Figure 13.3 Circulating neutrophils (PMNs) from injured patients who underwent initial resuscitation with either stored RBCs or PolyHeme. (a) PMN CD11/CD18 receptor expression in whole blood; (b) PMN superoxide production in isolated cells. * $P < 0.05$. (From: Johnson *et al.*, 2001, with permission.)

PMNs. The study groups (stored RBC [n = 10] versus PolyHeme [n = 9]) were comparable with respect to injury severity (ISS = 27.9 ± 4.5 versus 21.9 ± 2.7), physiologic compromise (emergency department pH = 7.22 ± 0.04 versus 7.19 ± 0.08), and Hb transfusion in the first 24 hours (units = 14.1 ± 2.0 versus 14.5 ± 1.0). Circulating PMNs from patients resuscitated with stored RBCs manifested evidence of priming through increased CD11b/CD18 expression and enhanced superoxide production (Figure 13.3). All patients in the PolyHeme group survived; three (30 per cent) in the stored RBC group died of MOF.

To further investigate the impact of early resuscitation with PolyHeme in lieu of stored RBCs, we extended our clinical trial to evaluate the systemic levels of pro-inflammatory cytokines (IL_6, IL_8), counter-regulatory cytokines (IL_{10}, IL_{11}), and markers of endothelial activation (sICAM, sE-selectin) (Johnson *et al.*, 2003). The study groups (stored RBC [n = 7] versus PolyHeme [n = 18]) were comparable with respect to injury severity. Patients resuscitated with stored RBCs had higher levels of the pro-inflammatory cytokines IL_6 and IL_8, and higher levels of the counter-regulatory cytokine IL_{10} (Figure 13.4), with a trend toward higher sICAM, and sE-selectin levels.

We have not enrolled a sufficient number of injured patients to definitively address the ultimate study objective – reduction of post-injury MOF. However, the incidence of MOF in the acutely injured patients given PolyHeme during their initial resuscitation for whom we had complete data (n = 20) was 15 per cent, contrasted with a predicted incidence of 36 per cent ($P < 0.05$) based on our MOF conditional probability model (age > 55 y., ISS > 25, RBC equivalent units > 6 in first 12 h 0–12 h base deficit <–8 meq/l, 12–24 h lactate >2.5 mmol/l) (Moore, 2003).

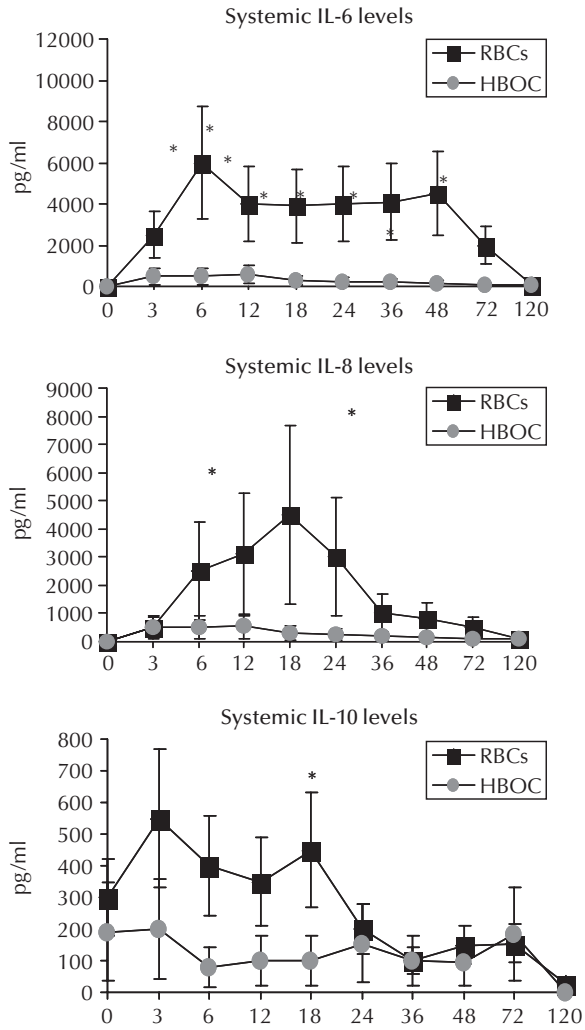

Figure 13.4 Systemic interleukin IL_6, IL_8 and IL_{10} from injured patients who underwent initial resuscitation with either stored RBCs or PolyHeme. (a) IL_6; (b) IL_8; and (c) IL_{10}. $^*P < 0.05$. (From Johnson *et al.*, 2003, with permission.)

In sum, these clinical trials in trauma patients suggest that PolyHeme, used in the early resuscitation of patients with hemorrhagic shock, will attenuate the immunodysfunction associated with stored RBC transfusion and thereby reduce the risk of post-injury MOF.

RBC non-availability: acute hemorrhagic shock

The most compelling indication for HBOC is the scenario in which stored RBCs are unavailable. This potential benefit for military use has largely

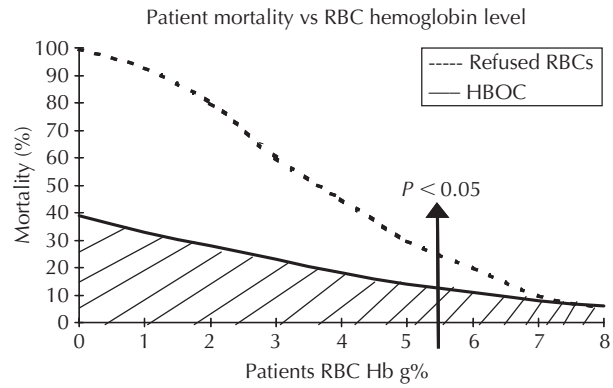

Figure 13.5 The 30-day modality is compared in surgical patients who refused stored RBC transfusion versus injured patients who were initially resuscitated with PolyHeme. Mortality was significantly less in the PolyHeme group when RBC Hb $\leqslant$ 5.3 g per cent. $^*P < 0.05$. (From Gould *et al.*, 2003, with permission.)

driven the development of HBOCs, but there are also a number of key applications in civilian trauma care. Most conspicuous is the role in pre-hospital care, particularly for extended transport times. However, there are also remote hospitals throughout the country in which stored blood is simply not available or is rapidly depleted when multiple casualties are encountered. There have been well-designed animal models that suggest pre-hospital low-volume resuscitation with HBOCs can save lives. Despite the appeal, the scientific design and ethical conduct of clinical trials to establish the efficacy of HBOCs when RBCs are unavailable remain a challenge.

To best approximate this scenario, we compared the 30-day mortality in 171 trauma patients given up to 20 units (1000 g) of PolyHeme, compared with a historic control of 300 surgical patients who refused stored RBCs on religious grounds (Gould *et al.*, 2002). The trauma patients received rapid infusion of 1 to 2 units (n = 45), 3 to 4 units (n = 4.5), 5 to 9 units (n = 47), or 10 to 20 units (n = 34) of PolyHeme; 40 patients had a nadir RBC Hb $\leqslant$ 3 g/dl (mean = 1.5 $\pm$ 0.7 g/dl). Total Hb was adequately maintained (mean = 6.8 $\pm$ 1.2 g/dl) via plasma Hb added by PolyHeme. The 30-day mortality in the PolyHeme group was 25.0 per cent (10 of 40 patients), compared with 64.5 per cent (20 of 31 patients) in the control patients (Figure 13.5). Additional case reports (Cothren *et al.*, 2002; Gannon and Napolitano,

2002; Lanzkron *et al.*, 2002; Mullon *et al.*, 2002) support the conclusion of this analysis.

A personal experience with PolyHeme has convinced us the time has arrived for the FDA approval of HBOC for trauma care (Moore, 2003). An 18-year-old man arrived by ground ambulance at our emergency department in extremis after a gunshot wound to the abdomen with a high-velocity elk-hunting rifle (30.06, hollow soft point 220 g, muzzle energy 2840 ft/lb). Because of immediate availability, 10 units of PolyHeme were administered during the first 14 minutes of in-hospital resuscitation, representing greater than 91 per cent of total circulating Hb at end infusion (RBC Hb = 0.7 g per cent). The missile entered the left midabdomen and exited directly posteriorly. At laparotomy, we encountered an avulsed shattered left kidney with secondary aortic and vena caval perforations, a partially transected superior mesenteric vein, and destructive injuries to the distal duodenum, proximal jejunum, mid-ileum, and descending and sigmoid colon. In addition, the patient had massive soft tissue loss in the retroperitoneum, including the psoas and paraspinous muscles, and suffered a concussive spinal cord lesion with resultant paraplegia. The patient received an additional 40 units of packed RBCs during initial laparotomy, but ultimately this gentleman survived to discharge without organ failure. We believe the immediate infusion of this HBOC was pivotal in maintaining sufficient oxygen delivery during the critical period of massive blood loss to save this man's life.

With this background, we initiated a large multicenter pre-hospital trial in the US in January 2004. Severely injured patients (blunt or penetrating injuries) with an SBP $\leqslant$ 90 mmHg due to acute blood loss are randomized at the scene to receive either the standard crystalloid resuscitation or PolyHeme. The study is conducted, by necessity, with Exception to Informed Consent. In the hospital, for the initial 12 hours post-injury the control group receives stored RBCs as needed while the study group is administered PolyHeme up to 6 units and then stored RBCs as needed. The primary study endpoint is 30-day mortality; the secondary endpoints include incidence of ARDS and MOF as well as amount of stored RBC transfusion.

THE NEXT GENERATION OF HBOCs FOR SURGICAL CARE

The potential efficacy of HBOCs for acute blood loss expands well beyond the temporary replacement for stored RBCs. Hemoglobin solutions might ultimately prove superior in delivering oxygen to ischemic or injured tissue (see Chapter 17). An indisputable byproduct of the intense competition to license HBOCs for clinical use is the enhanced knowledge of the fundamental physiology of hemoglobin. The current generation of HBOCs can save lives today; the next generation may be biochemically tailored for specific clinical indications (see Chapter 10).

EDITOR'S SUMMARY

Perhaps the most pressing need for a blood substitute is in unanticipated hemorrhage, in the operating room, on the battlefield, or on city streets. There is general consensus that restoration of blood flow and tissue oxygenation in the immediate post-injury period would lead to significantly better tissue salvage and, most likely, patient survival.

The use of a blood substitute in this setting would have the obvious advantages of immediate availability and no possibility for disease transmission. Furthermore, there is growing evidence that hemoglobin-based oxygen carriers might not only actually be superior to red blood cells in this setting, but also safer with regard to immunosuppression and cytokine activation.

One trial in trauma patients with tetrameric hemoglobin (HemAssist) was unsuccessful. A second major trial with a polymerized hemoglobin (PolyHeme) is currently under way, but no results are available at this writing. The design of clinical trials in trauma pose special ethical problems of informed consent, and require a high degree of safety of the product under study. Such clinical trials are also constrained by the fact that injured patients are heterogeneous, rendering outcome analysis challenging.

ACKNOWLEDGMENTS

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Chapter 14

Clinical Trials in Cardiac Surgery

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INTRODUCTION

Cardiac surgery is associated with significant blood transfusion and postoperative morbidity (Thurer, 2001; Chassot *et al.*, 2004). Tremendous efforts have been made to reduce these adverse events: special surgical techniques (Berson *et al.*, 2004; Puskas *et al.*, 2004), cell salvage (McGill *et al.*, 2002), restrictive transfusion protocols (van der Linden *et al.*, 2001; Ootaki *et al.*, 2004), anti-fibrinolytics (Porte and Leebeek, 2002) and artificial oxygen carriers have all been evaluated.

In cardiac surgery, cardiopulmonary bypass (CPB) is used when the heart is being arrested intraoperatively, to maintain circulation of oxygenated blood. The CPB circuit is composed of tubing, reservoir, oxygenator, pump and filters, and has a volume of up to 1500 ml in adults. It is normally pre-filled ('primed') with crystalloids. When connected to the patient this results in a distinct hemodilution with a corresponding decrease in hemoglobin and in the oxygen transport capacity. The use of artificial oxygen carriers to compensate for this decrease in oxygen transport capacity thus appears logical, despite the fact that oxygen demand is reduced by hypothermia. Indeed, several clinical trials on the use of artificial oxygen carriers in cardiac surgery with CPB have been recently published.

HEMOGLOBIN SOLUTIONS

Diaspirin crosslinked hemoglobin (DCLHb) in cardiac surgery

DCLHb is a modified hemoglobin in which the α-subunits are internally crosslinked with DBBF (see Chapters 30 and 33), produced as a 10 g/dl solution (Chatterjee *et al.*, 1986; Winslow, 2003). This prevents rapid breakdown of the hemoglobin molecule into $\alpha\beta$ dimers and results in an intravascular half-life of approximately 24 hours. It also decreases oxygen affinity and thereby increases *P*50 to 32 mmHg (Chatterjee *et al.*, 1986; Vandegriff *et al.*, 1989).

The first clinical study on the use of an artificial oxygen carrier in cardiac surgery was the DCLHb in cardiac surgery trial performed in 1995 and 1996 in 209 patients (Lamy *et al.*, 2000). Patients were randomized after cardiac surgery within 12 hours after removal from CPB, when presenting a prospectively defined transfusion indication, to receive either 250 ml of DCLHb or 1 unit of allogeneic red blood cells. These transfusion indications were a hemoglobin <7 g/dl or various forms of hemodynamic instabilities or co-morbid disease states at a hemoglobin >7.0 g/dl (Lamy *et al.*, 2000). When again presenting a transfusion indication, this treatment was repeated up to three times within the first 24 hours after CPB.

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In the DCLHb group, 59 per cent of patients avoided allogeneic red blood cell (RBC) transfusions until the first postoperative day, while, by study protocol, 100 per cent of patients randomized to the control group received allogeneic RBC transfusions. At hospital discharge, 19 per cent of patients in the DCLHb group had still avoided any allogeneic transfusion as compared to none in the control group. The use of DCLHb thus significantly reduced the exposure to allogeneic RBC transfusions in patients following cardiac surgery with CPB.

All patients experienced at least one adverse event (Lamy *et al.*, 2000). Serious adverse events were more frequent in the DCLHb group (50 vs 26 events), and more patients were affected (33 vs 21). The prevalence of the following adverse events was higher in the DCLHb group: hypertension, jaundice, increased liver and pancreatic enzymes, and hematuria/hemoglobinuria. Postoperative hemoglobin concentration was similar in both groups. Hemodynamically, DCLHb-treated patients tended to have higher systemic and pulmonary pressures and resistances and a lower cardiac output than patients treated with allogeneic RBCs.

A few years later, in 1997 and 1998, DCLHb was also used in trauma victims in the US and in Europe (Sloan *et al.*, 1999; Kerner *et al.*, 2003). Both studies were terminated prematurely due to an increased mortality in DCLHb-treated patients in the US study (Sloan *et al.*, 1999). The reason for the greater mortality in DCLHb-treated patients has not been found so far. There was clearly an excess of desperate cases in the DCLHb group in the US study, but this did not explain the difference in mortality (Sloan *et al.*, 2000). It might be hypothesized that a significant vasoconstriction resulting in a rise in blood pressure (Przybelski *et al.*, 1996) may have aggravated the blood loss in trauma victims and thereby compromised survival (Bickell *et al.*, 1994). As a consequence, the further development of DCLHb stopped following these events (Lamy *et al.*, 2000).

PolyBvHb (HBOC-201)

HBOC-201 is a polymerized bovine hemoglobin solution in which the intermolecular link is provided by glutaraldehyde reacting with surface lysines of the hemoglobin molecule (Stowell *et al.*, 2001). A final filtration eliminates the majority of non-polymerized hemoglobin tetramers to minimize nitric oxide scavenging within arterial walls due to presumed extravasation of hemoglobin molecules (Keipert *et al.*, 1994). The *P*50 of HBOC-201 has been reported to be 38 mmHg; it is manufactured as a 13 ± 1 g/dl solution, and has an intravascular half-life of approximately 24 hours (Krieter *et al.*, 1997; Levy *et al.*, 2002).

HBOC-201 has been used in cardiac surgery to reduce the need for allogeneic RBC transfusions (Levy *et al.*, 2002). In this trial, 98 patients were randomized at the first transfusion decision in the intensive care unit (ICU) following cardiac surgery. HBOC-201-treated patients avoided allogeneic RBC transfusions in 34 per cent of cases vs 0 per cent in the control group (as per study design). It is easy to understand why HBOC-201 treated patients had a lower hematocrit during the first three postoperative days, since HBOC-201 provides acellular (extracellular) hemoglobin. However, it is less readily understood why plasma hemoglobin was also significantly lower in HBOC-201-treated patients (Levy *et al.*, 2002). In the absence of formal transfusion triggers (Levy *et al.*, 2002), it might be speculated that patients in the HBOC-201 group were somewhat more restrictively transfused, explaining, in part, the difference in exposure to allogeneic RBC transfusions.

Adverse and serious adverse events were similar in the HBOC-201 and control groups (Levy *et al.*, 2002). Following HBOC-201 transfusion, increases in systemic and pulmonary artery pressures and a decrease in cardiac output were observed. Although not formally acknowledged in the paper, this indicates systemic and pulmonary vasoconstriction due to HBOC-201.

The side-effect profile of HBOC-201 has also been investigated in another study (Sprung *et al.*, 2002). In this study, escalating doses of 0.6–2.5 g/kg doses of HBOC-201 (corresponding to infusion volumes of 380 ± 87 ml to 1384 ± 309 ml) were given to 42 patients, and data were compared to those from 26 control patients receiving lactated Ringer's solution. Blood pressure was slightly but significantly higher in HBOC-201-dosed patients, a trend ($P = 0.06$–0.08) towards elevated postoperative lipase levels and a late methemoglobinemia (peak at the third postoperative day) were observed, and in 23 of 42 patients (58 per cent) IgG-antiHBOC-201 antibodies were detected at follow-up. In contrast, renal function, platelet count, blood coagulation parameters and general clinical laboratory values were similar in both groups (Spring *et al.*, 2002).

o-Raffinose polymerized hemoglobin

Hemoglobin raffimer is an o-raffinose crosslinked polymerized hemoglobin prepared from human hemoglobin (Ning *et al.*, 2000; Cheng, 2001; Hill *et al.*, 2002a). O-raffinose covalently crosslinks intramolecularly the α-chains to form a stable hemoglobin 64-kDa tetramer. In addition, o-raffinose reacts with surface amino acids of the intramolecularly stabilized tetramer to form polymers of 128–600 kDa. The final product is a mixture of ~40 per cent stabilized 64-kDa tetramers and ~55 per cent polymers. *P*50 has been reported to be 39 ± 12 mmHg, hemoglobin raffimer is manufactured as a 10 g/dl solution, and the intravascular half-life is approximately 24 hours (Cheng, 2001; Cheng *et al.*, 2004).

Hemoglobin raffimer has been evaluated in several studies in patients undergoing coronary artery bypass surgery (Hill *et al.*, 2002; Cheng *et al.*, 2004; Greenburg *et al.*, 2004). In these studies autologous blood was harvested by autologous intraoperative donation either immediately prior to (Hill *et al.*, 2002a) or during CPB (Cheng *et al.*, 2004; Greenburg *et al.*, 2004). The amount of blood removed was calculated so as to reach a target hemoglobin on cardiopulmonary bypass of 7 g/dl. Following autologous intraoperative donation and while on cardiopulmonary bypass, patients received either hemoglobin raffimer (250–1000 ml) or an equivalent dose of hydroxyethyl starch. In these studies, well-defined transfusion triggers were used. All autologous blood was returned to the patient either when a transfusion trigger was reached or towards the end of the procedure.

In the first phase II study, with 60 patients, the amount of RBCs transfused was less in hemoglobin raffimer-treated patients, but the percentage of patients completely avoiding allogeneic RBC transfusions was similar in both groups (Hill *et al.*, 2002b). The incidence of hypertension was higher and there was a greater postoperative increase in certain liver enzymes and lipase in hemoglobin raffimer-treated patients. In the second phase II study, again with 60 patients, a higher percentage of patients completely avoided allogeneic RBC transfusions (90 per cent versus 53 per cent, $P = 0.004$) (Cheng *et al.*, 2004). Hypertension and jaundice were observed as adverse events.

In a phase III trial, 299 patients were investigated. Hemoglobin raffimer-treated patients were exposed to allogeneic RBC transfusions in 56 per cent versus 76 per cent in hydroxyethyl starch-treated control patients ($P < 0.001$) (Greenburg *et al.*, 2004). Again, hypertension, jaundice, urine discoloration and a more pronounced increase in pancreatic enzymes were observed as adverse events.

PERFLUOROCARBON EMULSIONS

Perfluorocarbon emulsions are carbon–fluorine compounds characterized by a high gas-dissolving capacity (oxygen, carbon dioxide and other gases), low viscosity, and chemical and biologic inertness (Riess, 1992, 2001; Spahn *et al.*, 1994). Perfluorocarbons are virtually immiscible with water. Only by manufacturing an emulsion with specific characteristics (droplets of approximately 0.16 μm diameter) have they been rendered biocompatible, giving relatively few side effects. With the development of a stable 60 per cent (58 per cent perfluorooctyl bromide and 2 per cent perfluorodecyl bromide) emulsion, we now have a relatively highly concentrated emulsion which is clinically well tolerated (Riess, 1992, 2001; Wahr *et al.*, 1996; Keipert, 1998).

After intravenous application, the emulsion is taken up by the reticuloendothelial system. This uptake determines the intravascular half-life (Riess, 1992; Spahn *et al.*, 1994; Keipert, 1998), which is dose-dependent and is approximately 10 hours after a 1.8 g/kg perflubron emulsion dose (Riess and Keipert, 1998; Leese *et al.*, 2000). After the initial uptake of the perflubron emulsion into the reticuloendothelial system, the droplets of the emulsion are slowly broken down, the perflubron molecules are taken up in the blood again (bound to blood lipids) and are transported to the lungs, where the unaltered perflubron molecules are finally excreted via exhalation. At the present time, the metabolism of perflubron molecules in humans is unknown (Riess, 1992; Spahn *et al.*, 1994; Keipert, 1998).

Holman *et al.* (1995) tested perflubron emulsion in severely hemodiluted dogs undergoing CPB. Without using catecholamines, dogs treated with increasing doses of perflubron emulsion survived cardiopulmonary bypass progressively better than control animals. Subsequently, in 1996 and 1997, Hill and colleagues assessed the use of perflubron emulsion in 36 patients undergoing coronary artery bypass graft (CABG) surgery (Hill *et al.*, 2002b). All the patients underwent pre-CPB acute normovolemic hemodilution (ANH)

to a target hematocrit of 20–22 per cent during CPB. After initiation of CPB, patients received 1.8 g/kg or 2.7 g/kg perflubron emulsion, or 3 ml/kg saline in the control group. During CPB, the patients were monitored at 15-minute intervals for the presence of a physiologic transfusion trigger such as a mixed-venous oxygen partial pressure (PvO_2) < 30 mmHg, a mixed-venous hemoglobin saturation (SvO_2) < 60 per cent, or a hematocrit <15 per cent. When a trigger was reached, autologous and eventually allogeneic RBCs were transfused. Following CPB, all autologous whole blood was re-transfused to the patient. Postoperative transfusion triggers were: $PvO_2 < 35$ mmHg, $SvO_2 < 65$ per cent, a hematocrit <24 per cent, and ST-segment changes suggestive of myocardial ischemia.

There was similar number of adverse events, and two patients in each group suffered from a serious adverse event (Hill *et al.*, 2000b). As described for non-cardiac surgery (Spahn *et al.*, 1999, 2002), platelet counts were transiently lower postoperatively, but this did not result in bleeding events or more platelet transfusions. Patients in the 2.7 g/kg perflubron emulsion group triggered less frequently than control patients, and there was a trend towards a higher transfusion avoidance rate and a lower number of RBC units transfused (Hill *et al.*, 2000b). However, owing to the limited number of subjects studied, this did not reach statistical significance.

In 2000, a large multi-center phase III study on the use of perflubron emulsion in CABG surgery was performed. The study was scheduled to enroll 600 patients. As in previous cardiac (Hill *et al.*, 2000b) and non-cardiac studies (Spahn *et al.*, 1999, 2002) with perflubron emulsion, autologous blood harvesting was performed intraoperatively. Initially, all patients underwent a moderate degree of ANH (performed by the anesthesiologist) to reach an on-bypass hemoglobin of 8 g/dl. Patients randomized to receive perflubron emulsion received a dose of 1.8 g/kg following completion of ANH. These patients then underwent additional rapid autologous blood harvesting by the perfusionist when going onto CPB (targeted to achieve an on-CPB hemoglobin of 6 g/dl) and received an additional dose of perflubron emulsion (0.9 g/kg) thereafter. Protocol-defined on-CPB transfusion triggers as $PvO_2 < 30$ mmHg in hypothermic CPB and $PvO_2 < 35$ mmHg in normothermic CPB, $SvO_2 < 65$ per cent, ST segment changes suggestive of myocardial ischemia, and a hemoglobin <5.5 g/dl in perflubron emulsion-treated patients and <7 g/dl in control patients. In the postoperative period, a hemoglobin <8 g/dl served as a transfusion trigger in both groups.

Enrollment was voluntarily suspended in 2001 due to an imbalance in cerebrovascular accidents (Alliance Pharmaceutical Corp. press release, 8 January 2001). Clinical experts agree that these events were not directly related to the perflubron emulsion used, but rather to the rapid blood harvesting procedure when going on CPB resulting in hypotension – as suggested by a more common use of vasopressors. As data from the study were being analyzed for a detailed safety evaluation, a higher incidence of postoperative bleeding requiring re-operation was also noted in perflubron emulsion-treated patients. In these patients, the duration of bypass and the volume of hetastarch used were notable independent risk factors for bleeding complications. It is hypothesized that a dilutional coagulopathy due to the large volume of additional autologous blood harvested when going on-CPB and a greater volume of hetastarch used (in the perflubron emulsion-treated group), combined with the return of fully heparinized blood at the end of surgery, exacerbated the hemostatic dysfunction already caused by CPB. Interestingly, in the same study it was observed in a subset of patients monitored with gastric tonometry that gastric mucosal pH was higher in perflubron emulsion-treated patients, resulting in earlier postoperative return of normal bowel movement (Frumento *et al.*, 2002).

SUMMARY

In almost all studies in cardiac surgery, artificial oxygen carriers have reduced the need for allogeneic RBC transfusions and thus have proven their efficacy. Side effects have also been observed, although their clinical relevance is not entirely clear. Transient mild vasoconstriction after CPB (a period normally characterized by a marked vasodilatation) and transient skin and urine discoloration may not be overly serious adverse events. In contrast, cerebrovascular accidents, marked elevations of liver and pancreatic enzymes and postoperative bleeding are definitively undesirable. Cerebrovascular accidents and postoperative bleeding have been observed in protocols with rapid blood harvesting when going on CPB. This is a potentially risky procedure: it may cause immediate and severe hypotension

due to hypovolemia and the sudden drop in hematocrit, and the harvested blood is fully heparinized. At re-transfusion after CPB this may result in a re-heparinization of the patient with increased bleeding, unless additional protamine is given to neutralize the heparin. Autologous blood harvesting prior to going on CPB in the sense of traditional ANH thus may be the safer procedure since this blood is not heparinized. Alternatively, hemoglobin-based artificial oxygen carriers may be given only after CPB.

Progress in the development of artificial oxygen carriers has been made in recent years but no artificial oxygen carrier has yet achieved market approval in the US, Canada or Europe. Nevertheless, we should be anticipating the future development of such carriers and preparing for the necessary education and familiarization of healthcare professionals regarding these new concepts, physiology and compounds. Only in the hands of experienced professionals can artificial oxygen carriers be used to the benefit of patients.

EDITOR'S SUMMARY

Significant amounts of allogeneic blood are transfused in cardiac surgery, both intraoperatively, postoperatively, as well as to prime the bypass pump. Therefore these procedures offer attractive applications for blood substitutes. Several clinical trials in bypass procedures have been conducted, all with disappointing results.

These studies have demonstrated that blood substitutes can substantially reduce the use of allogeneic blood, but side effects have been found, some of which are serious. Transient vasoconstriction may be tolerated in this application in which vasodilation is a greater problem in the post-bypass period. However, trials with perfluorocarbon emulsions have resulted in significantly greater incidence of cerebrovascular accidents in the treated patients compared to controls – a consequence that cannot be accepted.

In reviewing this clinical experience, it is appreciated that the preoperative hemodilution procedure is a potentially risky procedure, emphasizing the need carefully to control the degree of hypovolemia and the rate of hemodilution. It appears that more experience and skill may be needed to perform this procedure safely than was previously appreciated.

Perhaps the best use of a blood substitute in cardiac surgery might be in the immediate post-bypass procedure. Alternatively, solutions may be useful in non-bypass procedures where timing and hemodynamic stability are critical.

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Chapter 15

Hemodilution

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INTRODUCTION

Hemodilution essentially indicates the proportional reduction of all cellular and plasmatic constituents of whole blood. Spontaneous hemodilution is the result of transcapillary refill after hemorrhage and trauma, as originally described by L. E. Gelin in his treatise 'Studies in anemia of injury' (Gelin, 1956). Hemodilution will necessarily take place when blood losses are replaced by asanguineous fluids, as first reported by the Swiss surgeon Kronecker (1886). He studied the ability of 0.6 per cent saline solution to replace blood and found that all his dogs tolerated an isovolemic exchange of blood as long as hematocrit was not dropped below 15 per cent. This was the first successful study on extreme hemodilution, since hematocrit (Hct) was reduced well below 20 per cent, which traditionally marks the borderline value between moderate and severe/extreme hemodilution.

With the aim of determining the tolerable degree of hemodilution, a great number of studies have been undertaken in dogs using crystalloid and colloidal solutions. These studies have been summarized by Messmer *et al*. (1972). They have shown that the hemoglobin concentration can be significantly reduced from its normal range while being compatible with long-term survival, provided that normovolemia is maintained throughout the period of dilutional anemia. Messmer *et al*. (1967) showed that isovolemic exchange of whole blood for the colloid Dextran 60 to a hemoglobin concentration of 2.8 g/dl was survived by all dogs studied. Takaori and Safar (1966) had already demonstrated that dogs survived dilution to a hemoglobin concentration of 3 g/dl, when isovolemically diluted with 6 per cent Dextran 75.

MECHANISMS OF ADAPTATION TO REDUCED HEMOGLOBIN

The experimental studies on intentional hemodilution or dilutional anemia have revealed that maintenance of a normal circulating volume is the absolute prerequisite for the tolerance and survival of significantly reduced hemoglobin concentration. While moderate hemodilution was survived by animals diluted with lactated Ringer's solution (RL) when the diluent was given at a volume 2.5 or 4 times the amount of blood withdrawn, extreme hemodilution was only tolerated safely when colloids were used as diluents (Messmer *et al*., 1972).

Viscosity-dependent increase in cardiac output

By 1956, Fowler *et al*. had already studied the hemodynamic effects of anemia with and without

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plasma volume expansion. In spontaneously breathing dogs undergoing isovolemic hemodilution with Dextran 75 to Hct values of between 18 and 11 per cent, they observed a mean increase of cardiac output by 53 per cent. Most of the subsequent studies confirmed that cardiac output increases with the reduction of Hct not only in the animal but also in human volunteers and patients. Cardiac output increases during normovolemic hemodilution, with the extent of the response being closely related to the actual decrease in whole blood viscosity. The increase of cardiac output is achieved mainly by an increase in preload and stroke volume, and only to a small extent by an increase in heart rate (Spahn *et al.*, 1994; Kreimeier and Messmer, 1996, 2002).

The reduction of whole blood viscosity is the key event in allowing the higher stroke volume, since the dilution of blood and the associated fall in blood viscosity induce a reduction of viscous resistance and thus of total peripheral resistance to flow associated with an increase of venous return (preload). The reduction in blood viscosity was considered to be the only mechanism responsible for the noted effects; however, the discovery of shear-dependant release of nitric oxide has contributed to the better understanding of the cardiovascular response to dilutional anemia (Doss *et al.*, 1995). Factors impeding the compensatory increase of cardiac output are conditions preventing an increase of venous return (hypovolemia, coronary artery disease preventing a dilution related enhancement of coronary blood flow, cardiac failure, and presence of negative inotropic factors or beta-blocking agents). Anesthesia may alter cardiac loading conditions, exert a negative inotropic effect and depress the autonomic nervous system, thus preventing an adequate cardiac output response (van der Linden *et al.*, 1994).

Myocardial perfusion and contractility

Normovolemic hemodilution to a hematocrit of 20 per cent leads to a preferential increase of coronary blood flow, as shown experimentally with electromagnetic and ultrasonic flowmeters as well as with microspheres. Since coronary blood flow rises out of proportion to the rise of cardiac output, coronary sinus PO_2 does not fall to critical values. Accordingly, measurements of local myocardial tissue PO_2 values showed unimpaired oxygenation of the myocardium during moderate hemodilution in the healthy animal (Forst *et al.*, 1987).

In patients undergoing acute normovolemic hemodilution (ANH) without signs of coronary disease, there were no signs of myocardial hypoxia. Even in patients with coronary heart disease, Laxenaire *et al.* (1986) reported absence of deterioration of myocardial perfusion scans (dipyridamol-thallium) before and after limited hemodilution. When compared to undiluted control patients, Baron *et al.* (1988) did not observe adverse effects of hemodilution to a target Hct of 25 per cent in anesthetized patients with coronary heart disease applying ECG (Holter tapes) and transesophageal echocardiography for control of ventricular function during the perioperative period. However, the authors stress that adequate cardiac monitoring is the prerequisite for ANH in this type of patient.

Enhanced myocardial contractility upon hemodilution has been observed by various authors over the years. This phenomenon has been corroborated by Habler *et al.* (1996), using load-independent variables. Enhanced myocardial contractility seems to contribute to the compensatory increase in cardiac output. In the same study, Habler *et al.* determined myocardial oxygen consumption. While the increased work of the heart in generating a higher cardiac output would suggest an increase in myocardial oxygen consumption, myocardial VO_2 was not significantly different from control values prior to ANH to 7 g/dl hemoglobin. It would appear that under conditions of reduced left ventricular afterload (viscosity-dependent fall of systemic vascular resistance and NO-mediated vasodilation), stroke volume rises without increase in myocardial VO_2.

Oxygen extraction response

The second adaptational mechanism in ANH is the increase in oxygen extraction from the diluted blood. It has been demonstrated by various groups that this mechanism comes into play when the cardiac response is inadequate to match the oxygen needs at the tissue level (van der Linden, 2001). Coronary blood flow is close to maximum at an Hct of 12 per cent; below this level there is the danger of development of myocardial ischemia, since oxygen extraction from coronary blood is almost complete already under normal conditions (Levy *et al.*, 1993). It should be noted that the oxygen extraction response may be limited by the presence of acute respiratory distress syndrome (ARDS) or systemic inflammatory response syndrome (SIRS), by traumatic

injury, ischemia-reperfusion syndrome or vasodilating drugs. In this situation, use of 100 per cent inspired oxygen fraction may improve the patients' tolerance to severe hemodilution, since hyperoxic ventilation significantly increases the delivery of oxygen dissolved in plasma to the tissues (Habler and Messmer, 1998; Habler *et al.*, 1998a).

From the above it is clear that under conditions of dilutional anemia the first organ at risk is the heart, and therefore monitoring of myocardial function becomes increasingly important the lower the level to which hematocrit is reduced (Kreimeier and Messmer, 1996, 2002). Nevertheless there are other organs to be considered, even though, with the exception of the coronary vascular bed, the elevated cardiac output is nearly proportionally distributed within the intact circulatory system. Owing to its relatively high oxygen extraction and the high total oxygen consumption, the gastrointestinal tract is the second organ at risk. About 75 per cent of intestinal blood flow is delivered to the mucosal/submucosal layer and only 25 per cent to the muscularis. Oxygenation of the mucosa during hemodilution is preserved by increased extraction of oxygen rather than by an increase of bulk intestinal flow (Panes *et al.*, 1992; Kleen *et al.*, 1996). Underperfusion of the mucosa has the potential to elicit damage to the muscosa barrier function, and thus renders the gut prone to translocation of endotoxins and bacteria with the well known sequelae of SIRS development (Kemming *et al.*, 2004a).

During moderate ANH, the redistribution of bulk flow away from the intestines may be compensated by enhanced intestinal O_2 extraction. It is well known that – similar to the heart – the capacity of the intestinal mucosa to increase tissue O_2 extraction is limited. In extreme anemia, microcirculatory intestinal O_2 shunts occur. These O_2 shunts are not due to an impaired capacity of the red blood cell to unload O_2; they are rather elicited by a decreased deformability of red cells leading to a reduced intestinal capillary perfusion (Schwarte *et al.*, 2005).

At the critical hematocrit, oxygen supply to tissue becomes inadequate and tissue hypoxia develops. Due to the limited O_2 extraction capacity, the corresponding Hct value may be higher for mucosa than for the heart (van Bommel *et al.*, 2001; van der Linden, 2001). Equivalently, the critical Hct may also be higher in the brain.

Therefore it is essential to generate normovolemia in order to avoid underperfusion and hypoxia of the gut, and tonometric measurements of mucosal pH are recommended for monitoring of gut perfusion and oxygen supply (Kleen *et al.*, 1996).

MICROCIRCULATORY CHANGES AND TISSUE OXYGENATION

When the hemoglobin concentration and thereby the oxygen content of arterial blood is reduced, the normal oxygen supply to the tissues can be preserved if: (1) there is a compensatory increase in flow rate; (2) oxygen unloading at the tissue level is enhanced by lowering of the venous PO_2; (3) there is a shift of the oxygen hemoglobin dissociation curve to the right; and (4) tissue oxygen consumption is reduced (by hypothermia or pharamacologically). All these mechanisms are known to be operational in chronic anemia. Regarding acute dilutional anemia, the importance of these mechanisms depends upon the grade or degree of hemodilution more than upon the kind of diluent used, provided normovolemia is maintained. Under moderate hemodilution, oxygen extraction remains unchanged as long as cardiac output and organ blood flow rise in relation to the decreasing hematocrit. Since oxygen extraction from coronary blood is almost complete under normal conditions, myocardial oxygen supply and function become limited by the increase in coronary flow, reaching the maximum values at about Hct 12.5 per cent (von Restorff *et al.*, 1975). Significant shifts of the oxygen dissociation curve, either due to loss of intraerythrocytic 2.3-DPG or as an effect of the crystalloid or colloidal diluent applied, have never been encountered during normovolemic hemodilution to Hcts of 10 per cent (Sunder-Plassmann *et al.*, 1974). Since at the second and third days 2.3-DPG and *P*50 values were found to be elevated, this phenomenon has been interpreted as an adjustment to the lowering of cardiac output towards the normal control level (Messmer and Sunder-Plassmann, 1974). The major local changes during ANH take place in the microcirculation.

Microcirculatory adaption to ANH

First of all it is of note that the microvascular hematocrit under normal conditions amounts to only approximately 50 per cent of the systemic arterial hematocrit, and remains in its normal

range as long as the systemic hematocrit does not drop below 17 per cent (Lindbom *et al.*, 1988); this indicates that the red cell concentration in the nutritional capillary vessels remains relatively constant. As a consequence, the amount of oxygen transported to the tissue becomes dependent upon the local red cell flux, which in turn depends upon the red blood cell velocity. At the tissue level, the increase in the red cell flow velocity is therefore the critical adaptation mechanism to hemodilution.

This has been shown by model analyses performed by Mirashemi *et al.* (1987) and experimentally confirmed for various tissues by Intaglietta and colleagues (Intaglietta, 1989). This group has provided evidence from animal experiments, with measurement of red blood cell flux in single capillaries of mesentery, muscle and skin, that red blood cell flux increases in these tissues up to a 50 per cent hemodilution. Below this value the regulatory mechanisms that control capillary hematocrit are no longer functional and arteriolar dilatation may take place, depending upon the organ and the type of arterioles (Lindbom and Arfors, 1985; Tigno and Henrich, 1986a, 1986b). Recent studies have implied a role of endothelial dilating factors (nitric oxide, prostacyclin) in the circulatory response to ANH, adding the factor of vasodilatation to the effect of reduced blood viscosity (Buga *et al.*, 1991; Doss *et al.*, 1995). Increase of local shear stress as a result of enhanced flow velocity results in the release of dilatory substances from the healthy endothelial wall, not only in macro- but also (and particularly) in microvessels. The dependence of this phenomenon on blood and plasma viscosity has been studied by Frangos and Intaglietta and their respective colleagues, and is discussed in detail in the Chapter 8.

In addition to the increase of red blood cell flux, hemodilution with colloid (Dextran 70) also enhances flow motion and thereby improves the distribution of red blood cells within the microvasculature (Mirhashemi *et al.*, 1987). The fundamental mechanism for all the microcirculatory changes discussed is the fact that the increase in flow velocity is directly related to the lowered blood viscosity (improved blood fluidity), the increase in cardiac output and the redistribution of local pressure in the microvessels, as demonstrated in the hydraulic model analysis of Mirashemi *et al.* (1987).

The overall result of the systemic and local adjustments to the lower blood viscosity is the maintenance of the normal functional capillary density (Menger *et al.*, 1988). Provided that normovolemia is provided and achieved through isovolemic exchange of blood for the colloid Dextran 60, Funk and Baldinger (1995) have shown that, in the hamster, moderate ANH provides unchanged functional capillary density (e.g. number of red cell perfused capillaries) while exchange of blood in a 4:1 ratio using RL was associated with a reduction of FCD, indicating the presence of capillaries without red cell flow – thus depriving the tissue of its source of oxygen supply.

TISSUE OXYGENATION

In agreement with the finding of maintained functional capillary density during moderate ANH, the local tissue oxygenation was found to be in the normal range of organ-specific tissue PO_2 values. Messmer *et al.* (1973) were the first to measure tissue PO_2 in dogs undergoing ANH by isovolemic exchange of blood for Dextran 60. Local tissue PO_2 was assessed polarographically with platinum multiwire electrodes – at the time the most sensitive method of detecting tissue hypoxia. Even though the hematocrit was reduced to 19 per cent, there was no evidence of tissue hypoxia occurring in the pancreas, small intestine, liver, kidney or skeletal muscle. PO_2 histograms showed that the frequency of low PO_2 values was not increased, and that the distribution of PO_2 values was more homogeneous and shifted towards higher values (Messmer *et al.*, 1973).

These results were pivotal for the application of ANH in clinical medicine, because they showed for the first time that moderate dilutional anemia does not jeopardize the oxygen supply to the tissues. Later studies confirmed the absence of tissue hypoxia for the myocardium and brain in animal experiments (Chan and Leniger-Follert, 1983; Forst *et al.*, 1987). While maintenance of the normal number of red blood cell perfused capillaries appears to be the basis for undisturbed tissue oxygenation, other mechanisms have been envisaged and experimentally approached. During states of hemodilution, red cells do not represent the sole source of oxygen to the tissues. Based on the original observation of a longitudinal gradient of oxygen along the arterioles (Duling, 1974), Intaglietta, Johnson and Winslow advanced the concept of oxygen leaving the blood from small arteries and arterioles prior to the red cells reaching the capillary bed (Intaglietta *et al.*,

1996). Measuring the specific localized oxygenation of segmental vessel walls and the perivascular tissue, the authors have shown that a significant amount of oxygen has already left the blood at the precapillary level; furthermore it was shown that the vessel wall consumes appreciable amounts of oxygen, which therefore do not reach the capillaries (Intaglietta *et al*., 1996; Intaglietta, 1997; Tsai *et al*., 1998a).

It should be noted that this phenomenon, namely precapillary oxygen loss, is reduced during hemodilution, and helps to maintain adequate tissue oxygenation. Due to the elevated red blood cell velocity, ANH reduces the loss of oxygen from the red cells with the effect that blood with higher oxygen content can reach the capillaries. Kuo and Pittmann (1988) and Kerger *et al*. (1995) have shown that red blood cells containing more oxygen will reach the most distal parts of the microvascular network. These findings corroborate the observations of Pries *et al*. (1992), who have elegantly demonstrated that hemodilution causes a redistribution of red blood cell flow in the terminal vascular bed with more red blood cells reaching the distal capillaries. A more homogeneous distribution of microvascular flow has also been reported by Tyml (1989), who studied not only the mesentery (as did Pries *et al*.) but also skeletal muscle.

BRAIN FUNCTION IN DILUTIONAL ANEMIA

In 1998 Weiskopf *et al*. started systematic studies in healthy unmedicated human volunteers. These authors confirmed the basic mechanisms of ANH as described above (Weiskopf, 2001) and, significantly, demonstrated that in healthy volunteers ANH to a hemoglobin concentration above 5 g/dl does not impair cognitive function, while subtle slowing of test responses to addition and digit-symbol substitution was registered at 5 g/dl hemoglobin (Weiskopf *et al*., 2000). More recently, the same group has shown that ANH to a nadir of 5.1 g/dl hemoglobin does not impair peripheral or central nerve conduction – a finding suggesting that anemia is not the cause for the slowing of cognitive function (Weiskopf *et al*., 2003).

PLASMA VISCOSITY

Based on hemorrheological considerations, for a long period of time it was assumed that elevated blood viscosity would impair microvascular function and therefore hamper the increase of capillary flow needed to compensate for the reduced oxygen content of the blood.

To clarify this issue, Brückner *et al*. (1993) performed experiments in which the diluent Dextran 60 was used as a 3 per cent and a 6 per cent solution for isovolemic hemodilution. Nutritional flow was assessed by means of radioactive microspheres, simultaneously with tissue PO_2 measurements. In the heart and liver the authors found no difference of flow response to ANH, even though the plasma viscosity of the diluents differed significantly. In an extension to these studies, Krieter *et al*. (1995) increased the plasma viscosity (from 1.06 cp to 2.99 cp) of animals simultaneously undergoing ANH to a hematocrit of 20 per cent. Measurements of nutritional blood flow were performed on the heart, brain, liver and skeletal muscle, while local PO_2 profiles were obtained from the liver and skeletal muscle. Neither the blood flow response nor the tissue PO_2 were diminished by the elevated plasma viscosity at significantly reduced hematocrit. Hence these authors concluded that small rises in plasma viscosity, as regularly observed when inducing ANH by use of colloid solutions (Dextran), will compromise neither the perfusion nor the oxygenation of vital organs in patients.

EXTREME HEMODILUTION

Extreme hemodilution is of interest not only for the delineation of the tolerable limits of dilutional anemia but also for clinical medicine, since patients who are Jehovah's Witnesses have to be treated without blood transfusions. Cooley *et al*. (1964, 1966) have introduced extreme hemodilution in Jehovah's Witness patients undergoing open heart surgery with extracorporeal circulation, by use of asanguineous priming fluids (Cooley *et al*., 1966). Conventionally, ANH is driven to a hemoglobin concentration of about 7 g/dl. This value defines the so-called transfusion trigger, and should not be exceeded in adult patients undergoing elective surgery when no additional protective measures (such as hypothermia, hyperoxia, use of oxygen carriers) are undertaken (van Woerkens *et al*., 1992). In the presence of coronary or congestive heart disease, ANH is generally not recommended (Kreimeier and Messmer, 1996, 2002). In otherwise healthy elderly patients, where compensatory mechanisms such as an

increase in cardiac output and/or heart rate do not fully come into play, higher hemoglobin concentrations might be recommended even in the absence of coronary or congestive heart disease (Spahn *et al.*, 1996; Weiskopf *et al.*, 1998). Young children, however, can tolerate lower hemoglobin values, as demonstrated in children undergoing the Harrington procedure in orthopedic surgery (Ott and Martin, 1981).

The fact that the conventional transfusion trigger (i.e., hemoglobin concentration of 7 g/dl) does not coincide with curtailment of the oxygen supply indicates that mechanisms other than oxygen must be operational in extreme hemodilution. According to Tsai and Intaglietta (2001), oxygen is not the critical deficit factor at the transfusion trigger, although below this level the risks for patients seem to be higher. Tsai *et al.* (1998b) introduced the concept of maintenance of functional capillary density by increasing plasma viscosity.

When hemodilution surpasses the transfusion trigger, the cardiac output does not further increase as blood viscosity is lowered. Tsai and Intaglietta (2001) explain this behavior by an increase of systemic vascular resistance via vasoconstriction, with the aim of maintaining the systemic blood pressure and thus autoregulatory perfusion of the vital organs. In experiments on hamsters bearing the skinfold window chamber allowing direct access to the microcirculation (Endrich *et al.*, 1980), Tsai *et al.* (1998b) measured functional capillary density under conditions of isovolemic blood exchange for Dextran 70 down to a hematocrit of 12 per cent. In contrast to moderate ANH, extreme ANH to this hematocrit (75 per cent blood exchange) was associated with a steep fall of FCD. This reduction in FCD could be completely reversed by increasing the plasma viscosity by means of high-viscosity Dextran 500 yielding a plasma viscosity of 2.8 cp, because this maneuver causes an increase of blood flow and at the same time restores FCD despite a hematocrit of only 12 per cent (Tsai *et al.*, 1998b).

The phenomenon of a normal FCD during extreme hemodilution is explained on the basis of the recent findings of shear stress-dependent release of dilating factors from the microvascular endothelium (De Wit *et al.*, 1997). Enhanced shear stress at the microvascular wall induces enhanced release of nitric oxide and prostacyclin from the endothelial cells, and thereby causes dilation of the larger arterioles feeding the capillary bed. The studies of Intaglietta and colleagues have stipulated the following explanation for how extreme hemodilution is tolerated. In extreme hemodilution the viscosities of blood and plasma have to be high enough to allow the local release of endothelial vasodilators, which counteract precapillary vasoconstriction, and the consequent reduction of FCD. These authors therefore call for a new class of hemodiluents when extreme hemodilution will be induced without jeopardizing the microcirculatory function. Such a beneficial effect of high plasma viscosity had already been demonstrated by Krieter *et al.* (1995); furthermore, De Wit *et al.* (1997) have shown, in microcirculatory experiments in the hamster cremaster muscle, that elevation of plasma viscosity indeed induces nitric oxide-mediated vasodilatation of precapillary arterioles.

HYPEROXIC HEMODILUTION

Hyperoxic hemodilution consists of the combination of extreme hemodilution and hyperoxic ventilation. This concept was introduced by Habler and Messmer (Habler and Messmer, 1998; Habler *et al.*, 1998a, 1998b) in order to allow the application of extreme hemodilution to patients undergoing elective surgery when high blood losses are anticipated. In such patients, when the conventional transfusion trigger is reached the patient is exposed to 100 per cent inspiratory oxygen while the ongoing blood loss is replaced by conventional resuscitation fluids. This technique allows the transfusion trigger to be further reduced, since oxygen is provided from the enlarged plasma compartment.

Of note, oxygen-induced vasoconstriction does not – as known from non-anemic animals and patients – cause deterioration in tissue oxygenation in extreme anemia. Although hemodilution-induced vasodilation is limited in this scenario (hematocrit 21 per cent), hyperoxic ventilation simultaneously augments oxygen content via the enhancement of diffusional oxygen transport by increased delivery of physically dissolved oxygen (Habler and Messmer, 1998; Habler *et al.*, 1998b), which finally outweighs the potential but unwanted effects of hyperoxic vasoconstriction on tissue perfusion. This also holds true when hemodilution is extended to the critical hematocrit value and peripheral tissue hypoxia and myocardial ischemia are present – hyperoxic ventilation is still able to restore myocardial, intestinal mucosal and peripheral tissue oxygenation, irrespective of concomitant vasoconstriction

(Kemming *et al.*, 2003, 2004a, 2004b), which may explain the improvement of survival following hyperoxic ventilation at the critical hematocrit (Meier *et al.*, 2004). The experimental data and the data from the first clinical application of this concept by Spahn *et al.* (1999) support the absence of hyperoxia-specific deregulation of microvascular perfusion in hemodiluted subjects. The practical applicability and safety of this concept will have to be proven in the future.

SUMMARY

Hemodilution induces macro- and microcirculatory responses, allowing the adaptation of the organism to the dilution-associated reduction in the oxygen content of the blood. While the cardiovascular response consists mainly of an increase in cardiac output and organ blood flow – normovolemia provided – the microcirculation responds with an increase of red blood cell velocity, more homogeneous distribution of microvascular flow, and well-maintained functional capillary density and tissue oxygenation. Extreme hemodilution is tolerated if the microcirculatory function is preserved by the elevation of plasma viscosity and thus higher shear stress on the microvascular wall. Hyperoxic hemodilution may be considered an effective method to make use of extreme hemodilution in the clinic. Hemodilution appears to be the most satisfactory technique to study the efficacy and safety of oxygen-carrying solutions applied as diluents.

EDITOR'S SUMMARY

Hemodilution is the practice of intentionally removing red blood cells to lower the hematocrit. This has the obvious result that the red cells can be replaced later if needed. However, the practice was first used to improve circulatory hemodynamics: lowered viscosity can increase the venous return to the heart and thereby raise cardiac output.

The question of how much the hematocrit can be reduced without causing detrimental effects has been addressed in animals and human studies. The conclusion seems to be that in the absence of disease the optimal hematocrit may be around 20 per cent, but that levels as low as 5 g/dL can still be tolerated at sea level in healthy volunteers.

Since the heart has a high metabolic demand for oxygen, and since the coronary sinus (venous) PO_2 is so low that oxygen extraction is almost maximal under normal conditions, the heart is likely to be the organ most at risk from intentional hemodilution. The concept that reduced blood viscosity is beneficial at moderate hemodilution may not apply at extreme hemodilution, where local shear forces are necessary to maintain release of nitric oxide, a vasodilator. Maintenance of these shear forces may be possible at very low hematocrit if the hemodiluent has relatively increased viscosity.

The role of supplemental inspired oxygen is also under investigation, even though its use has been considered routine for decades. Oxygen is a vasoconstrictor under normal conditions, but in extreme hemodilution, where dissolved oxygen is a larger proportion of total oxygen transport, it does not appear to have this effect. Further research on the combined and separate roles of nitric oxide, shear forces and oxygen will most probably clarify this interesting and important physiology in the near future.

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Clinical Hemodilution

Chapter 16

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INTRODUCTION

Hemodilution signifies a dilution of all normal blood constituents occurring either spontaneously after blood loss and tissue injury by the mechanism of capillary refill or intentionally as result of plasma replacement therapy or plasma expansion with crystalloid or colloidal infusion solutions.

In clinical practice, two different types of hemodilution have to be differentiated:

1. Acceptance of dilutional anemia as result of blood and plasma replacement with acellular fluids resuscitation shock therapy.
2. Intentional hemodilution resulting from blood removal and replacement of the blood withdrawn by crystalloid and colloidal solutions, with the aim of gaining and saving autologous blood for intra- or postoperative transfusion.

Acute preoperative hemodilution has been proposed by our group as a possibility for transfusing autologous blood (Laks *et al.*, 1973; Bauer *et al.*, 1974; Klövekorn *et al.*, 1974a, 1974b). The procedure has been applied in patients with anticipated major blood loss.

The first clinical studies of intentional hemodilution were based on a large series of experimental studies exploring the physiology and safety aspects of intentional preoperative anemia (Messmer *et al.*, 1972). These experimental studies have clearly shown that maintenance of normovolemia is the absolute prerequisite to elicit physiologic compensatory mechanisms ensuring adequate blood and oxygen supply to the tissues, despite a lowered hemoglobin concentration and hence oxygen content of the blood.

Acute normovolemic hemodilution (ANH) essentially means removal of whole blood from a patient while maintaining that patient's initial circulating volume by the infusion of an appropriate volume of crystalloid and/or colloid. Originally ANH was conceived as an alternative to donor blood, and to reduce the risks of exposure to allogeneic blood and potential transfusion reactions.

From the very beginning of implementation of clinical ANH, a main argument for the procedure was the availability of whole blood containing all cellular and plasma factors allowing transfusion at the end of operation, thereby improving the coagulation status of the patient.

Another benefit, anticipated from the animal experiments and in particular the microcirculatory studies with intentional hemodilution, is the improvement of tissue perfusion and tissue oxygenation due to the decrease in whole blood viscosity and the ensuing consecutive increase in

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nutritional blood flow (Messmer *et al.*, 1972). ANH has been recommended by the NIH Consensus Panel (1995) and the American Society of Anesthesiologists (1996) as an inexpensive and effective means for reduction of allogeneic blood exposure. However, the efficacy of ANH has recently been questioned. Bryson *et al.* (1998) have published a systematic review on the efficacy of ANH, including the clinical studies published until the end of August 1996. More recently, Segal *et al.* (2004) have published a meta-analysis of clinical studies published between 1972 and 2002, comparing ANH versus standard care. According to the authors, the literature supports only modest benefits from preoperative ANH, while the safety of the procedure remains unproven.

In fact, ANH has not reached the criteria of evidence-based medicine (EBM); nevertheless, it should be considered for patients undergoing elective surgery with anticipated major blood loss.

INDICATIONS FOR ACUTE NORMOVOLEMIC HEMODILUTION

From the very beginning, preoperative hemodilution was intended to counteract the increasing shortage of homologous blood and to avoid its immanent risks. Transfusion of allogeneic blood is expensive and, although safer than ever before, is still associated with complications such as clerical error, transfusion-related bacterial and viral infections, and immunosuppression with enhanced cancer occurrence.

Preoperative hemodilution is indicated in patients with anticipated blood losses >1500 ml (equivalent to 30 per cent of blood volume). The prerequisites for safe hemodilution are a preoperative hemoglobin concentration of 12 g/dl (under conditions of normovolemia), a normal ECG and normal myocardial function (no signs of ischemia, ST-segment elevation/depression, absence of unstable angina pectoris, absence of cardiac insufficiency and presence of ejection fraction >50 per cent) (Kreimeier and Messmer, 1996, 2002). Patients with restrictive and/or obstructive lung disease are not suitable for hemodilution, since adequate oxygenation of hemoglobin during passage of the pulmonary vessels might be hampered. It should be noted, however, that hemodilution may enhance pulmonary capillary blood flow. As a result, arterial oxygen content has been found to be elevated after hemodilution in several studies. In the presence of ventilation/ perfusion mismatch and increased left-to-right shunt flow, the oxygen content of arterialized blood may actually become reduced (see Table 16.1).

Table 16.1 Criteria for selection of patients for perioperative hemodilution

Estimated blood loss >1500 ml
Preoperative hemoglobin concentration >12 g/dl
Normal ECG and normal myocardial function (no signs of ischemia, no ST-deviation, no unstable angina pectoris, no congestive heart failure, no ventricular ejection fraction $<50\%$)
Absence of restrictive and/or obstructive lung disease (preoperative chest X-ray, respiratory functional testing)
Absence of renal disease (normal serum urea/ creatinine, no oliguria; no signs of single kidney dysfunction)
Absence of untreated hypertension (systolic arterial pressure >160 mmHg, diastolic arterial pressure >100 mmHg)
Absence of liver cirrhosis (plasma clotting factors within reference, serum albumin within reference)
Absence of coagulation disorders (*ín vivo* clotting time, plasma clotting factors within reference, no history of hereditary coagulopathy, platelet count $>150\,000/\mu$l)
Absence of infection (no fever, no leucocytosis/penia, no systemic inflammatory response syndrome, no invasive local infection)

Modified from Kreimeier and Messmer, 2002.

With hemodilution, all the cellular and plasma components of blood decrease in parallel and coagulation factors may be critically low. Therefore, pre-existing coagulation disorders are clear contraindications for ANH. Advanced age is no contraindication, unless associated with coronary heart disease (Vara-Thorbeck and Guerrero-Fernandez, 1985). Infants and small children (aged 1–8 years) have undergone preoperative ANH without any signs of impaired tissue oxygenation (Aly Hassan *et al.*, 1997; Jonas *et al.*, 2003).

Concerning general compensatory mechanisms for dilutional anemia, there is general agreement that only under conditions of normovolemia and increased preload hemodilution an adequate compensation of reduced hemoglobin concentration can be elicited and the adequacy of tissue oxygenation guaranteed.

PHYSIOLOGICAL COMPENSATORY MECHANISMS

Cardiac output

In 1968 Hint predicted that by linear lowering of the hematocrit, systemic oxygen transport capacity, i.e., total oxygen supply (arterial oxygen content × cardiac output), would first increase slightly, then reach a peak at approximately 30 per cent hematocrit, and would not fall below initial control values as long as the hematocrit did not exceed 20 per cent (Hint, 1968). This particular bell-shape of the systemic oxygen transport capacity curve was explained by the exponential fall of whole blood viscosity, and was basically confirmed *in vivo* by the experiments of Sunder-Plassmann *et al*. (1971) on healthy dogs undergoing isovolemic exchange of blood with Dextran 60 or packed red cells, varying the hematocrit between 70 and 10 per cent. Hence, ANH to hematocrit not lower than 20 per cent is associated with maintenance of total oxygen transport capacity in its control range, indicating a wide range of safety in terms of unimpaired total oxygen supply. It should be noted that this feature of the oxygen transport capacity curve has normovolemia and normal cardiac function as absolute prerequisites.

In principle, the shape of the oxygen transport capacity curve predicted by Hint (1968) has been corroborated by *in vivo* experiments by Krieter *et al*. (1995) and by the model analyses performed by Duruble *et al*. (1979) and Mirhashemi *et al*. (1987). In addition, reports in the literature contain oxygen transport capacity values higher than control in patients (Laks *et al*., 1973; Martin *et al*., 1987). Hence normovolemic dilutional anemia down to hematocrits of 20 per cent (moderate hemodilution) does not seem to compromise the systemic oxygen supply in healthy individuals.

Tissue oxygenation

The first evidence that moderate dilutional anemia does not compromise tissue oxygenation was presented by Messmer *et al*. (1973) by simultaneous measurements of local tissue PO_2 in the liver, pancreas, kidneys, small intestine and skeletal muscle. Despite the deliberate reduction of the hematocrit to 20 per cent, the tissue PO_2 values were found to be normal or slightly increased in all the organs studied. Meanwhile, similar data have been reported for the brain and heart (Chan and Leniger-Follert, 1983; Forst *et al*., 1987). Brain function was found to be slightly reduced (in responses to tests of addition and digit symbol substraction) in unmedicated healthy human volunteers diluted isovolemically to 5 g/dl hemoglobin (Weiskopf *et al*., 2000).

Tissue oxygenation is enhanced by an increase of red blood cell velocity in the capillaries due to improved fluidity of the blood. Today it is well accepted that functional capillary density, i.e., the number of red cell perfused capillaries, is well maintained during normovolemic hemodilution, and the oxygen supply remains fully adequate by this microcirculatory adaptation to the reduced number of circulating red cells, despite significant reduction of hematocrit (Messmer *et al*., 1988; Tsai *et al*., 1998; see also Chapter 7).

HEMODILUTION PROCEDURES

Calculation of blood volume withdrawal

The efficacy of hemodilution depends upon the baseline and the target hematocrit chosen, as well as upon the magnitude of intraoperative blood loss. In order to reach the desired target hemoglobin concentration or hematocrit, the formula developed by Bourke and Smith (1974) is usually applied to determine the volume of blood to be withdrawn; however, it should be noted that this formula systematically overestimates the truly exchangeable blood volume (EBV). If this was the case, the actual degree of anemia might be more severe than intended, thus endangering patients. Meier *et al*. (2003) have developed a new mathematical model for correct prediction of exchangeable blood volume during acute normovolemic hemodilution. While the target hemoglobin concentrations of 7 and 9 g/dl were overestimated by 15 per cent in dogs and 20 per cent in patients when using the Bourke–Smith formula, the new interactive model predicted EBV more reliably (overestimation in dogs 1 per cent, in patients 8 per cent). This new interactive approach helps to improve patient safety and provides an adequate physiological basis for further studies to explore the efficacy of ANH and the avoidance of allogeneic blood transfusions.

Practical aspects of hemodilution

Preoperative hemodilution is performed either before or immediately after induction of

anesthesia. Induction of hemodilution following that of anesthesia has gained widespread acceptance because it is easier to achieve hemodynamic stability of the patient under anesthetics, and in general there is enough time to dilute the patient to the desired target hematocrit prior to significant blood loss. It is obligatory to obtain consent of the patient for preoperative hemodilution. In Europe, the procedure should be offered when the probability of transfusion of blood or blood products amounts to >5 per cent. Autologous blood must be withdrawn under aseptic technique, and the autologous blood must be adequately stored. The activity of clotting factors will be diminished by 50 per cent after 4 hours, and will have ceased after 6 hours at room temperature. If storage time exceeds 6 hours, cooling at 4°C is mandatory in case delayed retransfusion is desired (possible <12 h). If the patient's blood is not retransfused during this time it has to be discarded; this procedure has to be part of the patient's consent.

Technique of preoperative hemodilution

Knowledge of the patients' initial hematocrit and body weight and of the target hematocrit is essential for calculation of the volume to be withdrawn for acute normovolemic hemodilution. The greater the difference between the initial and target hematocrits, the greater the efficacy in terms of transfusion avoidance. Furthermore, the efficacy of the procedure depends upon the amount of intraoperative blood loss. After determination of the exchange volume, blood is withdrawn from either a venous or an arterial line connected to a three-way stopcock into standard blood collection bags containing anticoagulant, usually CPDA (50 ml). The bags containing the autologous blood are marked with the patient's name, numbered sequentially and stored at room temperature. They should be gently agitated at times. A movable spring balance appears the best means of quantifying blood withdrawal and the amount of replacement fluid. Usually the blood bags should be filled with exactly 450 ml of autologous blood to ensure the correct concentration of additives and avoid accidental clotting. For this reason, a special bag should be used for children (maximum filling volume 250 ml). Simultaneously with blood withdrawal, crystalloid and/or colloidal solutions are infused at the same speed to ensure maintenance of the circulating blood volume. Withdrawal and diluent infusion are terminated when the envisaged target hematocrit is reached. Depending upon the trigger hematocrit for transfusion, the autologous blood is retransfused, always beginning with the most recently obtained unit. By this procedure, the first unit of autologous blood withdrawn (which is richest in red cells, platelets and clotting factors) is infused last, preferably at a time when the surgical bleeding has been controlled (retransfusion in reverse order of removal). The acceptable intraoperative limits according to the German guidelines (6 g/dl in healthy subjects and 8–10 g/dl in patients with pre-existing cardiovascular disease; Bundesärztekammer, 2001) serve as the basis for setting the transfusion trigger. In general, the autologous blood should not be retransfused before the institutional transfusion trigger has been reached. It is widely accepted that patients' ability to compensate for anemia is interindividually different, depending on the actual oxygen requirements, the cardiovascular reserve, the depth of analgesia sedation and any coexistent inflammation. Therefore, the data regarding the hematocrit threshold at which tissue hypoxia and myocardial ischemia occur may vary considerably. The decision as to whether retransfuse the precollected blood is related to whether tissue hypoxia is imminent at a given Hct (Hardy, 2003; Marshall, 2004). Therefore, the patient's response to anemia is monitored to enable timely retransfusion – which of course requires adequate monitoring techniques, enabling online bedside-assessment.

Monitoring

In clinical practice, the level of invasiveness of monitoring procedures will depend on the degree of hemodilution (Kreimeier and Messmer, 2002). In healthy patients with a target hematocrit of 20–25 per cent, continuous monitoring of the ECG (non-invasive), systemic blood pressure, central venous pressure, pulse oximetry and urinary output appears sufficient for the patient's safety, and intermittent controls of deviations from the target hematocrit are mandatory. Intermittent control of arterial blood gases and laboratory parameters including coagulation status are recommended (Table 16.2).

When greater blood loss and therefore more profound hemodilution is envisaged, the monitoring has to become more invasive. It is recommended that the systemic blood pressure be monitored continuously via an arterial line.

Table 16.2 Hemodilution: monitoring variables in the perioperative period

Hemodilution to Hct 20–25%		Hemodilution to Hct below 20–25%	
Continuous monitoring	Intermittent monitoring	Additional (highest priority)	Facultative (acc. to target Hct)
ECG-monitor (lead II or V)		ECG-monitor (lead II and V)	On-line ST-segment analysis
Arterial blood pressure (invasive)		Arterial blood pressure (invasive)	Pulmonary arterial catheter (CO, PCWP, continuous S_vO_2)
Central venous pressure (curve display)			Arterial pulse contour analysis (CO, GEDV, SVV)
Pulse oximetry	Blood gas analysis (PaO_2, SaO_2, BE, pH, Hb, CaO_2)	Blood gas analysis; central venous or mixed venous (PvO_2, SvO_2, CvO_2)	O_2 transport indices (DO_2I, VO_2I, O_2 ER)
Urinary output	Cumulative urinary output (hourly)		
	Blood chemistry (K^+, Cl^-, Na^+, Ca^{++}, blood glucose, coagulation status, fibrinogen, platelet count)	Blood chemistry (arterial lactate, serum albumin)	Bedside Hb-test (Hemocue), thrombelastography

PaO_2 = arterial O_2 partial pressure; SaO_2 = arterial hemoglobin O_2 saturation; BE = base excess; pH = pons hydrogenium; Hb = arterial hemoglobin concentration; CaO_2 = arterial O_2 content; K^+ = potassium concentration; Cl^- = chloride concentration; Na^+ = sodium concentration; Ca^{++} = calcium concentration; PaO_2 = (mixed) venous O_2 partial pressure; SvO_2 = (mixed) venous hemoglobin O_2 saturation; C_vO_2 = (mixed) venous O_2 content; CO = cardiac output; PCWP = pulmonary capillary wedge pressure; GEDV = global end-diastolic blood volume; SVV = stroke volume variation; DO_2I = global O_2 delivery; VO_2I = whole body O_2 consumption; O_2ER = O_2 extraction ratio.
Modified from Kreimeier and Messmer, 2002.

The maintenance of normovolemia is necessary to preserve tissue perfusion and oxygenation. Formerly, insertion of a Swan–Ganz catheter was the method of choice for assessment of left ventricular filling and cardiac output (thermodye-method). Today it is known that static parameters of cardiac preload, such as central venous pressure or pulmonary wedge pressure, are not very sensitive in distinguishing between normo- and hypovolemia. During hypovolemia, the cardiac stroke volume is reduced. According to the Starling curve of the ventricle, a patient requires fluid administration unless further fluid loading will not result in a further increase of stroke volume or cardiac output. Recently, a new method – arterial pulse contour analysis – has been proposed for monitoring the volume status of the patient. This method is capable of showing whether a mechanically ventilated patient responds with a rise in stroke volume upon IV fluid administration (hypovolemic patient), or whether there is no response (normovolemic or hypervolemic patient). This 'non-invasive' technique requires an arterial line and an additional central venous line, and allows the measurement of ventilation-induced changes in stroke volume, cardiac output and global end diastolic cardiac filling volume (Reuter *et al.*, 2003).

In cases where hemodilution is extended to very low Hcts, a closer monitoring of oxygen transport becomes necessary. Classically, placement of a pulmonary artery catheter allows collection of mixed venous blood. From mixed venous blood gas values, the oxygen transport indices (including the oxygen extraction of the tissues) can be deduced, which may help to detect borderline compensation for ANH. Recent data suggest that oxygen hemoglobin saturation measured in the superior vena cava is closely correlated to mixed venous oxygen saturation, which holds true under normal conditions and also during reduced oxygen supply (Reinhart *et al.*, 1989;

Ladakis *et al.*, 2001). This renders monitoring of oxygen transport feasible without the considerable risks of pulmonary arterial catheterization.

When the limit of possible hemodilution is approached, continuous monitoring techniques should be used. Fiberoptic methods allow continuous measurement of mixed venous SO_2, allowing the best online assessment. This technique still requires a pulmonary catheter. Continuous ST-segment analysis (preferable via leads II and V5) is easy to apply, and is clinical standard procedure today.

Intraoperative imaging by means of transesophageal echocardiography (TEE) will provide additional information regarding cardiac filling and performance. However, it requires an experienced investigator (Kreimeier and Messmer, 2002). Tachycardia during hemodilution calls for meticulous analysis of the underlying reason and instant control by adequate measures, including – in cases of doubt – timely retransfusion (Kreimeier and Messmer, 2002).

EFFICACY OF HEMODILUTION

The efficacy of ANH is still a matter of debate. Over the years there have been many published studies, retrospective and prospective, which have reported very different results – ranging from significant or total avoidance of allogeneic blood to total lack of efficacy. The variation in the results is certainly due to different study protocols, degrees of ANH (target hematocrit), types of surgical procedures (from minor surgery up to liver resection and liver transplantation, and cardiac surgery with ECG) and the type of diluent used, but also to the choice of outcome parameters.

The meta-analysis by Segal *et al.* (2004) considered controlled studies on hemodilution carried out between 1972 and 2002. Despite the well-known notion that the efficacy of ANH depends upon the baseline and the predetermined target hematocrit as well as the magnitude of intraoperative blood loss, Segal *et al.* included in their meta-analysis studies with baseline hematocrits between 43 and 36 per cent, while the target Hct values ranged from 30 to 18 per cent. This would indicate that a stringent stratification of the studies was not intended, as also reflected in the inclusion of surgical procedures both with and without extracorporeal bypass. It is therefore not surprising that the authors came to the conclusion that ANH offers modest benefits in terms of avoidance of allogeneic blood, with unproven safety. The authors have called for large, carefully controlled prospective randomized studies before ANH can be endorsed for general use in elective surgical patients.

Notwithstanding this, there have been very recent publications that report significant blood savings in different surgical procedures (Jonas *et al.*, 2003; Jovic *et al.*, 2003; Casati *et* al., 2004; Habler *et al.*, 2004; Jabbour *et al.*, 2004). In particular, the studies of Goodnough *et al.* (1994, 1999) and Monk *et al.* (1995) have illustrated the usefulness and cost-effectiveness of ANH as compared to preoperative autologous donation in patients undergoing radical prostatectomy.

While single authors or single centers with devotion to all the alternatives of transfusion avoidance strongly believe in the value of ANH as part of blood sparing programs (Jonas *et al.*, 2003; Habler *et al.*, 2004; Jabbour *et al.*, 2004), a prospective multicenter study with well characterized criteria (baseline and target hematocrit, anesthesia, surgical procedure and type of diluent) should be undertaken to determine the true value of ANH.

ADJUNCTS TO ANH

Several efforts have been made and procedures suggested to further increase the efficacy of ANH and to improve the margin of safety concerning the development of tissue hypoxia. For all four of the following methods of increasing the efficacy of ANH, large prospective controlled studies should be performed.

Erythropoietin (EPO)

By preoperative treatment with erythropoietin (EPO) it is possible to increase the initial, predilutional hematocrit, allowing a wider difference between the starting hematocrit and the desired target value. This procedure requires specific preoperative logistics, since the increment of hemoglobin takes place only after a couple of days after beginning EPO therapy. However, the patient will then not present as being anemic at the time of surgery, and is therefore considered better off than patients undergoing preoperative collection of autologous blood. However, the use of EPO will significantly increase the costs of ANH (Goodnough *et al.*, 1989; Walpoth *et al.*, 1993).

Hyperoxia

Our group has suggested hyperoxic hemodilution to bridge the time until retransfusion of the withdrawn blood becomes necessary, due to the contribution of oxygen dissolved in the plasma during dilutional anemia (Habler and Messmer, 1998; Habler *et al.*, 1998a; Kemming *et al.*, 2003, 2004a, 2004b; Meier *et al.*, 2004). Of note is the fact that hyperoxic ventilation is not associated with vasoconstriction in the presence of low hemoglobin values (Habler and Messmer, 1998; Habler *et al.*, 1998a), whereas vasoconstriction regularly occurs when hyperoxia is induced at normal hemoglobin concentration, thereby impairing tissue oxygen delivery due to precapillary arteriolar constriction and reduced functional capillary density (Lund *et al.*, 1980; Sjoberg *et al.*, 1999; Tsai *et al.*, 2003).

Hypervolemia

Hypervolemic hemodilution (i.e., hemodilution after initial volume load in the euvolemic patient) was originally suggested by Kirchner (1971). In a recent publication, Kumar *et al.* (2002) compared preoperative isovolemia vs hypervolemia in surgical and orthopedic patients. They found isovolemic hemodilution to a Hct of 25 per cent by means of polygeline infusion did not differ from hypervolemic hemodilution in significantly reducing perioperative allogeneic blood requirements, time needed and costs incurred.

Blood substitutes

With the aim of extending the hemodilution to lower Hct-values and thereby increasing the efficacy of ANH, Habler *et al.* (1998b) made use of artificial oxygen-carrying compounds. The administration of artificial oxygen carriers during hemodilution is based on the following considerations. A patient scheduled for elective surgery first undergoes acute normovolemic hemodilution to the desired hematocrit. When the present transfusion trigger is reached during surgery, an artificial oxygen carrier (here a fluorochemical compound) is administered to enhance the oxygen content and thereby oxygen delivery to the tissues. As a result, the patient's hemoglobin concentration can be reduced to lower levels than under ANH without compromising tissue oxygenation. Upon retransfusion of the autologous blood, augmented hemodilution will provide a relatively higher hemoglobin concentration for the postoperative period. The advantages of this procedure are the higher gain of autologous blood and the more efficient avoidance of allogeneic transfusion, as shown experimentally by Habler *et al.* (1998b) and first demonstrated in surgical patients by Spahn *et al.* (1999). Later, this method was introduced as augmented ANH (A-ANH) (Spahn *et al.*, 2001).

SUMMARY

Hemodilution is a means of avoiding transfusion of allogeneic blood in patients undergoing elective surgery with anticipated major blood losses. Maintenance of normovolemia is the absolute prerequisite to achieve effective cardiovascular compensation for the intentional dilutional anemia. The target hematocrit and thus the degree of hemodilution can be calculated by various mathematical formulae. The occurrence of tachycardia in a diluted patient calls for immediate control of hematocrit, volume status and assessment of ST-segment changes in the ECG.

The efficacy of ANH in avoiding allogeneic blood transfusions depends on the initial hematocrit, the target hematocrit desired and the amount of blood loss. While age *per se* is no contraindication to ANH, patients with coronary disease, pulmonary disease or coagulation problems should not undergo the procedure routinely.

EDITOR'S SUMMARY

Hemodilution means reduction of the various components of the blood. In intentional hemodilution, blood is removed from a patient prior to surgery either to eliminate or to reduce blood transfusion (the blood can be returned later when needed), or to improve local circulation. Success of the procedure depends on the patient's ability to compensate for mild anemia, which includes elevated cardiac output.

The first objective, blood sparing, can be achieved in a significant number of cases. Patients must be carefully selected, however, by

strict criteria, including an adequate preoperative hematocrit, no serious impediment to oxygen transfer (normal cardiac and pulmonary function), and no risk factors for coronary ischemia. The second objective, maintenance or improvement in the microcirculation, is somewhat less well established in patients, although there is strong evidence from animal studies to support the concept.

An especially appealing aspect of the hemodilution procedure is its potential application with blood substitutes. It would seem that the ideal hemodiluent would be an oxygen carrier. However, to date no product has proven to be sufficiently without side effects to allow widespread application.

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Chapter 17

Potential for Blood Substitutes in Tissue Ischemia

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INTRODUCTION

Tissue ischemia is one of the most common causes of morbidity and mortality in the Western world today. About 50 per cent of the adult population is at the risk of having an ischemic episode during their life, leading to disability or death. The organs at highest risk are the heart, the brain, and peripheral tissues in the legs and arms. In this chapter the symptoms of ischemia in the legs will be discussed, along with the theoretical possibility of using plasma expanders with oxygen-carrying capacity to improve the ischemic situation of this region.

PERIPHERAL ARTERIAL OCCLUSIVE DISEASE

Peripheral arterial occlusive disease (PAOD) is a major cause of cardiovascular morbidity. The main reason for the arterial insufficiency is atherosclerosis, and one of the most common symptoms is intermittent claudication. The prevalence of this symptom is 1–2 per cent in the adult population, but the number of people who have asymptomatic disease is about three or four times higher than this. There is no major difference between men and women in this regard (Dormandy and Rutherford, 2000).

The most severe symptom in PAOD patients is chronic critical leg ischemia (CCLI), and the incidence is estimated to be about 500–1000 patients per million of the population per year. The fate of patients who have this serious symptom is very poor. Within one year after first presentation, 25 per cent of patients require a major amputation. After 5 years, only half of them are still alive and with two legs; 20 per cent are dead (Kannel and Ghee, 1984; Fowkes *et al.*, 1991).

Atherosclerotic lesions in the peripheral arteries are asymptomatic as long as the blood flow through the vessel is sufficient to support the tissues with oxygen and nutrients. Occasionally an artery can be occluded without the patient having any symptoms, because of a good collateral circulation in the region. However, when a stenotic artery becomes occluded, severe symptoms often occur. Such thrombotic occlusions are relatively common in the leg arteries. If stenoses or occlusions occur in the arteries above the knee region, intermittent claudication is often the result. If the obstruction affects the lower leg arteries, rest pain and skin ulcers or gangrene may be the final outcome.

Rest pain

One of the earliest symptoms in patients with CCLI is rest pain. This is predominantly localized

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to the most distal parts of the leg (i.e., the foot and toes). Usually the pain first becomes evident when the patient is in the supine position (e.g. in bed at night). How early in the disease process the pain appears depends on the severity and location of the arterial obstruction. The patient most often experiences relief if the lower leg is moved out of bed into a hanging position. This increases the hydrostatic pressure and improves the blood filling of the nutritional vessels in the ischemic region, resulting in pain relief. In severe cases the patient has to sit, stand up or walk around in order to relieve the pain. If the obstructions are marked, the pain will also be present in the sitting and standing positions, and it is then often intolerable in the supine position.

Ulcers and gangrene

The most severe signs of CCLI are ischemic ulcers or gangrene localized in the most distal parts of the leg, predominantly in the first, fourth and fifth toes (Conrad, 1968; Fagrell, 1973). The heel is sometimes also involved. The reason for this is almost certainly that these regions are most frequently exposed to trauma from shoes (Figure 17.1). In patients who have severely compromised blood supply to the foot, even slight pressure from a shoe may lead to abolition of skin microcirculation in the area, resulting in tissue necrosis (Fagrell, 1973). Consequently, it is of utmost importance that patients should be told to wear shoes that are large enough so that no pressure is applied.

The primary reason for the development of ischemic skin ulcers in patients who have CCLI is obstruction in the main arteries of the leg. However, necrosis of the skin does not occur until the blood flow in the nutritional capillaries of the ischemic region decreases to below that required for the minimal demand of oxygen and nutrients to the tissue. This is clearly demonstrated by the fact that only about half of the patients who have CCLI will develop skin necrosis in the ischemic foot during a 3-month observation period (Fagrell and Lundberg, 1984). The reason for this is that the nutritional demand of the skin is extremely low, and it can survive without nutrients and oxygen for days or weeks before it dies. If the nutritional capillaries are occasionally filled with blood, the tissues in a foot may survive. However, if red blood cells cannot enter the capillaries (see Figure 17.2), the tissue will sooner or later die. There are several possible reasons for this (Fagrell, 2004). It has been shown that microthromboses in 30–50-μm diameter vessels are very common in these areas (Conrad, 1968). Secondly, edema in the ischemic areas, especially in diabetic patients, may compress the nutritional capillaries so that no blood cells can enter. A third reason for blood not entering the capillaries in ischemic skin is that the pressure in the feeding arterioles is so low, the blood bypasses the capillaries and travels through less resistant shunt vessels (Fagrell, 2004).

In order to evaluate the nutritional status of the ischemic skin areas, microcirculatory techniques have to be used. The most sensitive and direct method is capillaroscopy. It can be used in

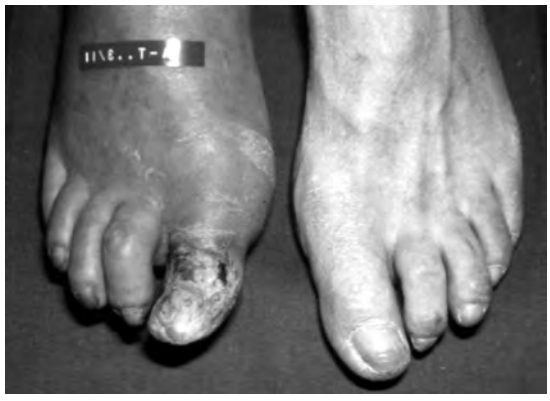

Figure 17.1 A patient with severe chronic critical leg ischemia (CCLI) in his right foot. See color plate 1.

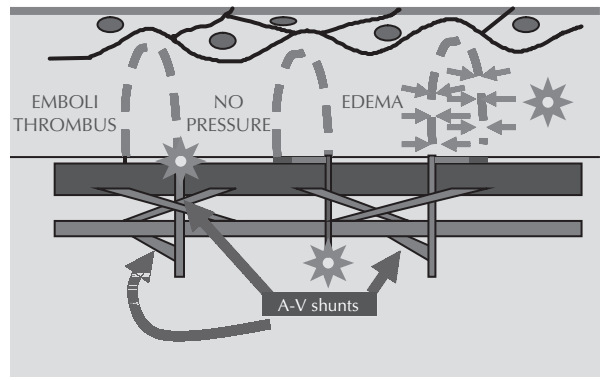

Figure 17.2 Schematic drawing of the skin microcirculation in an ischemic region. There are three possible reasons for blood cells not entering the nutritional capillaries: (1) microthrombo-emboli; (2) too low an inflow pressure to open the precapillary arterioles; and (3) interstitial edema compressing the arteries. Plasma can usually enter the capillaries. See color plate 2.

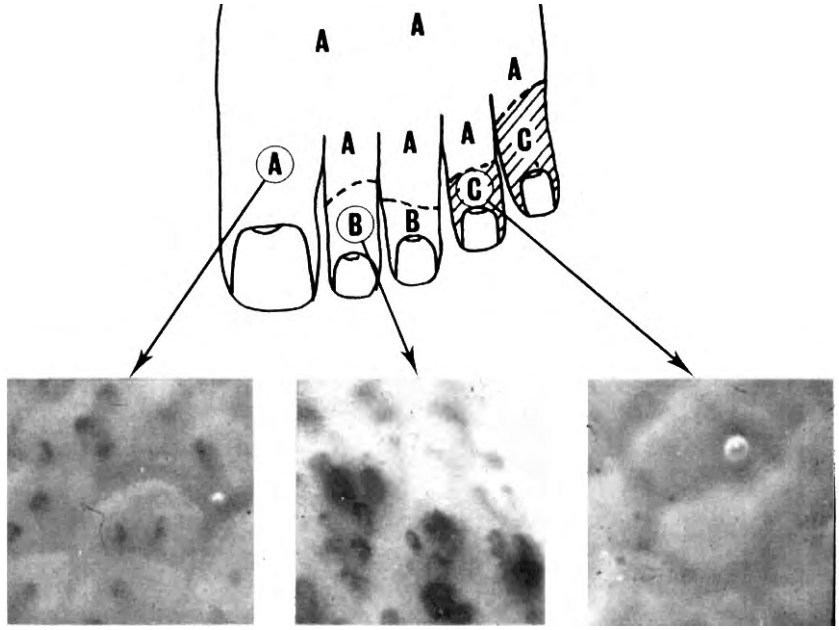

Figure 17.3 Schematic illustration of how the microcirculation can be affected in different regions of an ischemic foot. 'Stage A' represents an area where the capillaries are filled with blood, and there is no risk of necrosis. In 'Stage B', edema and capillary hemorrhages can be seen, indicating an ischemic destruction of the microcirculation. In the area indicated by 'Stage C', no blood-filled capillaries can be seen and the risk of necrosis is imminent.

clinical practice, and evaluates, directly and non-invasively, the blood filling and morphology of the nutritional skin. Because ulcers are most often localized to limited areas, such as a toe, it is necessary to use a technique that can map the microcirculation in all parts of the foot. This is possible with capillaroscopy (Fagrell, 2004).

It has been shown that if a normal structure and normal blood filling of the capillaries are seen in the skin (Stage A in Figure 17.3), the risk of necrosis developing is less than 10 per cent during an observation period of 3 months, regardless of the macrocirculatory status. However, if marked destruction of the capillary bed is present (Stage B in Figure 17.3) or if no blood enters the nutritional capillaries (Stage C in Figure 17.3) in the sitting position, there is an almost 100 per cent risk of skin necrosis developing over the same period. The technique has been shown to be very valuable in predicting the risk of skin ulcers in patients who have CCLI, and also for evaluating the prognosis of ischemic foot ulcers (Fagrell, 2004).

TREATMENT AVAILABLE FOR CHRONIC CRITICAL LEG ISCHEMIA

The aim of all treatments for patients with CCLI is to try to restore the blood supply in the nutritional capillaries of the ischemic region. This can be done in several ways, by both medical treatment and surgical procedures, and these are described below.

General management

Most patients with CCLI have other concomitant disease, especially in the cardiovascular and renal systems. Lung diseases, such as chronic bronchitis and bronchial carcinoma, also often occur, owing to the high proportion of smokers. It is therefore imperative to inform patients of the necessity to stop smoking. Several studies have shown that both the macro- and microcirculation of ischemic areas may be improved by smoking cessation. The reason for this is that the rheologic properties of the blood, the oxygen transportation by red blood cells and the blood viscosity all improve, resulting in enhanced nutritional skin circulation (Jonasson and Bergström, 1987).

Increase transmural pressure

As rest pain and ulcers are signs of severely impaired circulation in the nutritional skin capillaries, great efforts should be made to improve blood flow in these vessels (Bollinger and Fagrell, 1990). In order to improve the pressure

gradient across the vessel wall in the ischemic area, it is recommended that the ischemic foot be kept in the lowest possible position without inducing edema.

Improve the local microcirculation

Occlusions of the precapillary arterioles (30–50 μm diameter) and empty nutritional capillaries have been found to occur at increased frequency in the toes of patients who have CCLI (Conrad, 1968; Fagrell, 1973). Even the slightest pressure from a shoe may damage ischemic skin tissue and completely prevent blood from entering the nutritional skin capillaries, resulting in an ischemic ulcer (Figure 17.1). Consequently, efforts must be made to try to improve the opportunity for blood to enter these nutritional capillaries. There are several means of achieving this, including the following:

1. *Control of edema*. Edema in the ischemic area compromises the nutritional skin circulation and must be intensively treated. It has been shown that edema, especially in diabetic patients with CCLI, may totally compress the nutritional skin capillaries in ischemic areas so that they become completely void of blood (Fagrell and Lundberg, 1984). By reducing the edema in such areas blood may enter the capillaries, with a concomitant improvement and even healing of the ischemic ulcer, in spite of there being no improvement of the macrocirculation. However, treatment with diuretics may reduce the systemic blood pressure and this can be deleterious for the nutritional circulation in the ischemic region. Consequently, systolic blood pressure must be carefully monitored during treatment of edema.
2. *Revascularization procedures*. Most patients with CCLI have stenotic or occluding lesions of the leg arteries at multiple levels, and stenotic changes can be successfully treated with angioplasty in almost all patients who have iliac or femoral obstructions. This is the first-choice procedure, provided that a specialized interventional vascular radiologist or angiologist is available (Belli *et al.*, 1993; Dormandy and Rutherford, 2000). Limb salvage rates of more than 80 per cent after 2 years have been achieved with this procedure (Schwarten, 1991). Intra-arterial stents can be used in combination with angioplasty, and may improve the patency rate after this procedure.
3. *Thrombolysis*. Local coagulation must be avoided by, for example, the use of lytic agents such as streptokinase, urokinase or tissue plasminogen activator (TPA). This is now implemented routinely to open stenotic or occlusive obliterations in the leg arteries (Wholey *et al.*, 1998). The lysis can be performed systemically, but intra-arterial thrombolysis has emerged as the primary method. The procedure is most often combined with percutaneous transluminal coronary angioplasty (PTCA) of the underlying stenosis.
4. *Pharmacological treatment*. The main purpose of all treatment procedures in patients with CCLI is to improve the nutritional blood flow in the ischemic tissue. As the ischemic symptoms in these patients are primarily due to macrovascular obstruction, opening procedures should be performed if possible. If this cannot be done, then conservative treatment has to be implemented. The possibility of improving the nutritional blood flow in severe ischemic skin areas with pharmacological agents is limited because of the marked reduction in total blood flow to the region. Another reason why very few drug trials have shown any statistically significant improvement in CCLI is that it is difficult to collect a homogeneous group of patients if only macrocirculatory parameters are used to classify the patients. As noted above, microcirculatory methods must be used to determine the nutritional status in an ischemic skin area (Fagrell, 2004), and very few such studies have been performed. Nevertheless, remarkable positive effects of some vasoactive drugs can sometimes be demonstrated in single patients.

   The only drug therapy that has convincingly been shown to have a positive effect on rest pain and skin necrosis is intravenous infusions of prostanoids. The first studies were reported in the 1970s, with prostaglandin E_1 and prostaglandin I_2, but the results were inconclusive. More recently, stable prostacyclin analogs have been tested, and there is now good scientific evidence that these compounds have a positive effect in reducing rest pain and healing ulcers (Loosemore *et al.*, 1994).
5. *Antiplatelet drugs*. Reduction of platelet adhesion may be used for long-term treatment in order to reduce the progression of atherothrombosis in leg arteries, and it has been shown that these agents may also reduce vascular events such as stroke and myocardial infarction in about 25 per cent of

patients who have atherosclerotic disease (Rössner and Müller, 1987).

6. *Anticoagulant treatment*. Anticoagulation may also be considered in patients who have CCLI in order to reduce the risk of micro- or macro-thromboembolism in stenotic, atherosclerotic vessels.

Other treatments

Hyperbaric oxygen therapy and hemodilution have been tried, but there is no scientific support for these interventions. However, advances in molecular biology may generate new principles for the treatment of patients who have PAOD. For instance, in pilot studies, vascular endothelial growth factor (VEGF) seemed to promote angiogenesis in selected patients who have CCLI (Baumgartner *et al.*, 1998).

Amputation

Amputation may be the ultimate, but also the best, treatment for some patients who have CCLI when all other treatments have failed. It should be borne in mind that patients who have severe ischemia that makes amputation necessary also face an extremely poor long-term prognosis regardless of treatment. Only about half of these patients will be alive after 2–3 years (Dormandy *et al.*, 1988).

POTENTIAL FOR BLOOD SUBSTITUTES IN THE TREATMENT OF CCLI

As the primary cause of ischemic symptoms in the leg is obliteration of the main arteries, the first treatment should be to try to open up these vessels. If such a procedure is successful, the ischemic symptoms will in most cases be improved. However, as many of the patients suffering from CCLI are elderly and have concomitant disease, surgical procedures may not always be possible. In these cases other treatment procedures must be tried. The goal of all treatments is to improve the blood flow in the nutritional vessels of the ischemic areas, and, as described, this can be achieved by trying different non-invasive treatments. One such possibility may be to use oxygen-carrying plasma expanders.

Theoretical possibilities

In regions subjected to a reduced arterial circulation, the blood flow in the microcirculation is also successively reduced. However, it is not until the blood flow in the capillaries is totally eliminated that there is a risk of tissue death. This is especially the case in the skin, where the nutritional demand is extremely low. Skin tissue can survive for days without any red blood cells entering the capillaries. This has been clearly shown in many patients with CCLI by capillary microscopy (Figure 17.3). However, in most of these patients there is plasma flow in the capillaries, and consequently small particles and molecules (but not blood cells) can enter the capillaries of these ischemic areas. As most oxygen-carrying substances are relatively small molecules, they will easily enter the microcirculation where only plasma flow is seen. Consequently, there are good theoretical possibilities that these substances may improve the oxygenation of areas exposed to ischemia.

Improving functional capillary density

In one recent study, the oxygen-carrying blood substitute based on polymerized bovine hemoglobin (PolyBvHb) was used to determine efficacy in maintaining tissue PO_2 following an 80 per cent isovolemic blood exchange leading to very low hematocrit (19.5 per cent). One hour after exchange, arteriolar and venular flow were reduced from baseline. Arteriolar flow was also significantly lower 12 hours after exchange, but recovered after 24 hours. These results suggest the impairment of tissue oxygen metabolism following the introduction of PolyBvHb into the circulation, which is mitigated as the concentration of PolyBvHb declines (Cabrales *et al.*, 2004).

In another study it was investigated whether resuscitation with polyethylene glycol-modified human hemoglobin (MP4), an oxygen-carrying blood replacement fluid, could improve systemic and microvascular variables after hemorrhagic shock. Hamsters implemented with a skin-fold chamber were hemorrhaged 50 per cent of blood volume, and then resuscitated with 50 per cent shed blood volume, 5 per cent hydroxyethyl starch (HES), or MalPEG-Hb. The results showed that the presence of MalPEG-Hb improved microvascular blood flow and oxygen transport during shock to a significantly greater extent than that attainable with blood or HES (Wettstein *et al.*, 2003).

Both these experimental studies support the possibility that oxygen-carrying plasma expanders may be used in clinical practice for improving oxygenation in ischemic tissues. As

CCLI is a localized and severe ischemic disease, where both the macro- and microcirculation can be accurately determined by sophisticated methods, this is an ideal region for testing. The possibility of improving the situation for such patients with medication is also very limited. The treatment of choice is primarily PTCA or vascular surgery, but these treatments cannot be performed in many patients because of their poor clinical status.

In several animal studies performed with MP4 there have been strong indications that this substance improves the oxygenation of tissues by increasing the functional capillary density of the tissues (Tsai *et al.*, 2003). There are also theoretical grounds for the hypothesis that MP4 will be able to release oxygen to ischemic tissue better than blood can. The two main reasons for this are that the oxygen delivery is regulated by a shift in the dissociation curve, and that the molecule is so small it can enter capillaries where only plasma flow is possible. If this is proven to be the case, intriguing possibilities emerge regarding the use of MP4 as a treatment in patients with different kinds of ischemic conditions.

A critical issue is the half-life of the different oxygen-carrying substances. Some of them have a half-life of less than 12 hours, while MP4 has been shown to have a half-life of at least 24 hours. This should be beneficial in the treatment of ischemic tissues. However, in a chronic disease like CCLI, repeated infusions of the oxygen-carrying substance might be necessary to improve the situation for the patient.

SUMMARY

There are theoretical, experimental and clinical indications that oxygen-carrying substances may be of benefit for treating ischemic conditions in humans. A perfect substance should be small enough to be able to enter capillaries where only plasma flow is possible. It should also be able to keep the oxygen bound to the hemoglobin until it reaches the ischemic tissue, and then to deliver it in that tissue. The half-life should be at least 24 hours, and the substance should, of course, be non-toxic. The substances that at present appear to closest meet these demands are MalPEG–Hb solutions.

The potential for a solution that can improve the oxygenation of ischemic tissues is great. In acute situations, e.g. myocardial infarction or stroke, improving the oxygenation of the ischemic tissue region might be life-saving. If an oxygen-carrying substance with the properties mentioned can be given directly in the emergency room, as soon as the patient arrives, the area of tissue threatened by ischemia might be reduced and a greater amount of it kept alive until the obliterated artery has been opened up by, for example, revascularization procedures.

In order to show if a substance has these potentially beneficial effects in clinical practice, human studies have to be performed. The most attractive 'model' for this purpose seems to be the ischemic leg. The reason for this is that it is a chronic ischemic, and also often a rather stable condition. The macro- and microcirculation of the area can be easily and accurately measured with clinically available techniques. The local oxygenation of the tissue can also be easily measured with available methods. It is also possible to grade the local microcirculation and oxygen tension, and to find the area where the oxygenation is lowest. This will increase the possibility of observing an improvement by infusion of the substance in question.

EDITOR'S SUMMARY

Tissue ischemia is one of the greatest causes of morbidity, especially in Western cultures. The cause is vascular, but the effect of insufficient oxygen delivery to tissue is necrosis, or it can lead to stroke (brain), infarction (heart) or claudication (muscle). Cell-free oxygen carriers could be the ideal treatment for such disorders, at least acutely, but they must not engage vasoconstriction or else they could contribute to the pathology. Sickle cell anemia is a special case where the primary pathological event is cellular rigidity and failure to flow, but the result is the same: tissue anoxia. Oxygen carriers that can penetrate tissue better than blood could be very effective in treating these diseases.

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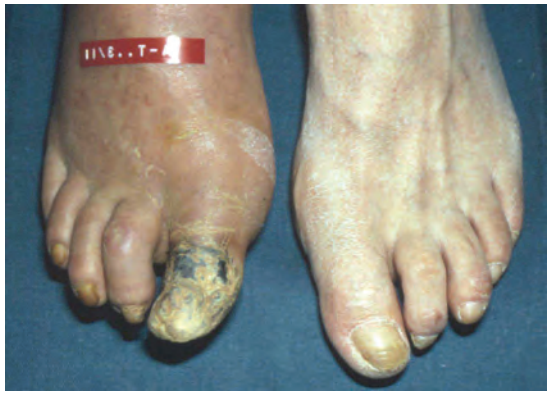

Plate 1 A patient with severe chronic critical leg ischemia (CCLI) in his right foot. See Fig. 17.1.

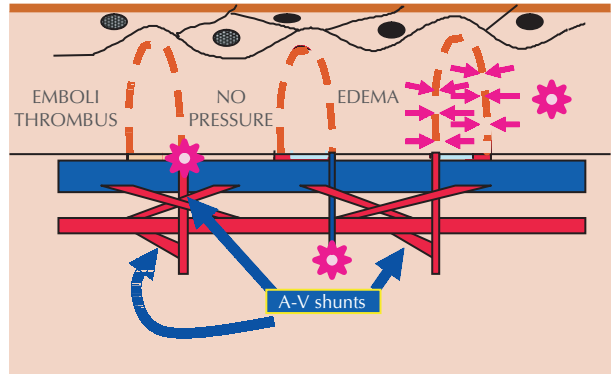

Plate 2 Schematic drawing of the skin microcirculation in an ischemic region. There are three possible reasons for blood cells not entering the nutritional capillaries: (1) microthrombo-emboli; (2) too low an inflow pressure to open the precapillary arterioles; and (3) interstitial edema compressing the arteries. Plasma can usually enter the capillaries. See Fig. 17.2.

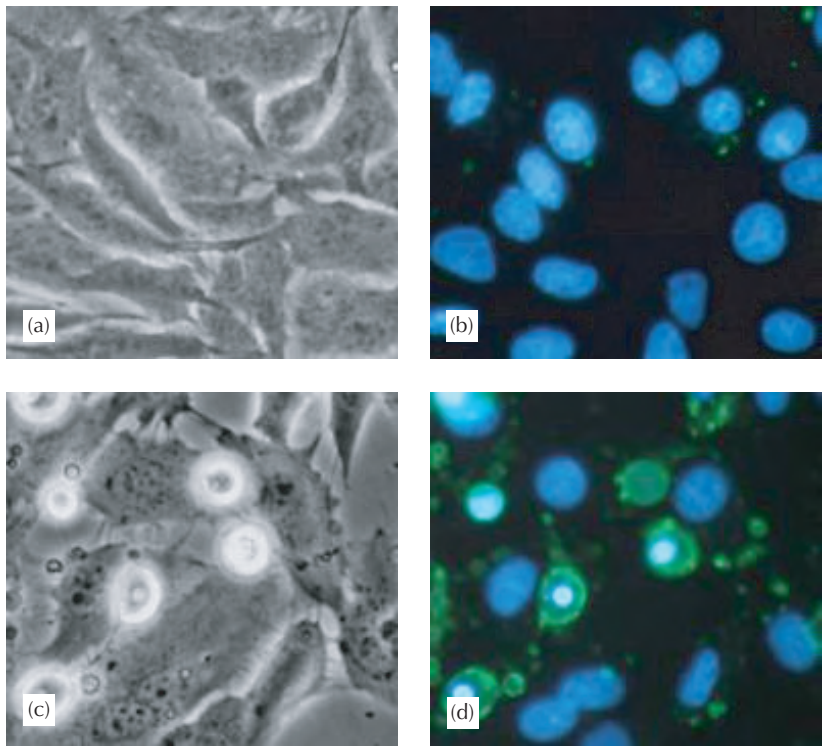

Plate 3 Redox cycling of αα-Hb causes endothelial cell cycle arrest and apoptosis. Bovine aortic endothelial cells were treated with (a, b) serum-free medium alone, or (c, d) medium containing 50 μM αα-Hb and H_2O_2-generating glucose oxidase. After 16 hours, cells were stained with Hoechst 33342, Alexa Fluor 488 annexin V conjugate, and propidium iodide (PI), and visualized by phase contrast and immunofluorescence microscopy (for detailed description see D'Agnillo, 2004). Hoechst 33342, a cell permeant blue fluorescent DNA dye, was used to visualize nuclear morphology. Alexa Fluor 488-annexin V conjugate was used to detect phosphatidylserine on the outer leaflet of the plasma membrane, an early apoptotic marker. PI, a red fluorescent nucleic acid dye, enters and stains cells with decreased plasma membrane integrity. Control cultures showed an intact monolayer with typical pale blue nuclei staining by Hoechst 33342 and no annexin V or PI staining (a, b). Treatment with αα-Hb and glucose oxidase caused cell rounding, detachment, and condensation of nuclei stained brightly by Hoechst coupled with annexin V plasma membrane staining (green), indicative of apoptosis (c, d). Glucose oxidase or αα-Hb alone did not produce these morphological changes (not shown). See Fig. 19.1.

SECTION

4

Toxicity and Side Effects

It is easily demonstrated that cell-free oxygen carriers can deliver oxygen to tissues. The main problem with their acceptance into clinical use has been their safety, including concerns over toxicity and side effects. Analysis of these effects is complex because animal models are not always good representations of human disease states, and so there is a constant feedback cycle between clinical trials and basic research. This section reviews some of the concerns, primarily with hemoglobin-based products, that are still unresolved. Studies involving critical organs at risk (kidneys, central nervous system, gut, heart and endothelium) are discussed in detail.

Chapter 18

Redox and Radical Reactions of Hemoglobin Solutions: Toxicities and Protective Strategies

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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.

INTRODUCTION

Several problems associated with transfusions of human donor blood have motivated the development of blood substitutes. HBOCs are designed to correct oxygen deficit due to ischemia, and are primarily developed for use in the emergency resuscitation of trauma patients and for perioperative hemodilution during surgical procedures (Reiss, 2001).

For hemoglobin to be fully operational in a cell-free environment, two problems must be overcome to ensure adequate oxygen supplies to tissues: the oxygen affinity of the hemoglobin may not be suitable for the acellular environment, and hemoglobin in dilute medium dissociates into $\alpha\beta$ dimers when infused and can cause serious kidney damage. Several site-specific and in some instances bifunctional reagents have been introduced to stabilize the hemoglobin molecule in a tetrameric or polymeric species with oxygen-binding characteristics that equal or surpass those of red blood cells (RBCs) (for review, see Reiss, 2001).

Documented adverse events with current-generation HBOCs include interference with normal blood pressure mechanisms, gastrointestinal and pro-inflammatory effects, oxidative stress, pancreatic and liver enzyme changes, neurotoxicity, and the development of heart lesions in some animal species. The mechanism for the pressor effect is not completely understood; however, the binding of hemoglobin to vascular endothelial-derived nitric oxide (NO) has emerged as a likely candidate (for review see Alayash, 1999, 2004). Experiences with these HBOCs have shown that there are a number of challenges that must be recognized and resolved before the clinical usefulness of these products can materialize. First, hemoglobin outside its natural protective environment of red blood cells is inherently toxic due to its uncontrolled oxidative side reactions. Second, the proximity of free hemoglobin to the vascular sources of nitric oxide may result in the creation of an oxidative milieu, to the detriment of both hemoglobin and the vasculature. Third, chemical modifications may in some cases actually enhance the ability of hemoglobin to undergo spontaneous and chemical oxidation.

The focus of this chapter will be on hemoglobin oxidative chemistry, and the possible contribution

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of these reactions to the overall toxicity of hemoglobin solutions along with the emerging strategies to control them.

OXIDATIVE MECHANISMS

Hemoglobin outside its protective red-cell environment undergoes unhindered oxidation of its iron center – a process known as autoxidation. Spontaneous oxidation of the ferrous/oxy derivative ($HbFe^{2+}$) leads to non-functional ferric heme ($HbFe^{3+}$) and a superoxide ion ($O_2^{\bullet -}$), which subsequently dismutates to generate hydrogen peroxide (H_2O_2). These species can ultimately damage the protein and/or the heme group. An essential intermediate in the pathway leading to this damage is the ferryl heme ($HbFe^{4+}$), itself formed through the reaction of the $HbFe^{2+}$ or $HbFe^{3+}$ with H_2O_2 (see Equations 18.1, 18.2). Accompanying the formation of ferryl heme is a protein/porphyrin radical cation ($^{\bullet}HbFe^{4+} = O$; Equation 18.2). In the process, reactive oxygen species (ROS) are generated which can further sustain a cycle of oxidative side reactions, ultimately leading to oxidative modification(s) of the protein and heme loss.

$$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+} = O + H_2O + O_2 \quad 18.1$$

$$HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+} = O + H_2O \quad 18.2$$

Normal intra-erythrocytic Hb exists in an environment rich in the enzymes catalase and superoxide dismutase, which can together effectively catalyze the breakdown of the $O_2^{\bullet -}$ and H_2O_2 produced by the spontaneous oxidation of the ferrous iron. Reductase systems, which catalyze the reduction of the $HbFe^{3+}$ back to $HbFe^{2+}$ (the only form that reversibly binds oxygen), are also present.

There is a considerable amount of published literature on the interactions of cell-free hemoglobins (both modified and unmodified) with H_2O_2, which is produced by hemoglobin oxidation or from other cellular sources (Everse and Hsia, 1997; Alayash *et al.*, 2001a). These complex reaction mechanisms have been shown to be dependent on the type of chemical modification(s) introduced to modify hemoglobin oxygen affinity, the initial oxidation state of the heme iron, and (probably) other factors. It is clear that, under appropriate conditions, damaging and potentially toxic species are formed, including the ferryl protein ($HbFe^{4+}$) – a highly reactive hemoglobin-associated oxidant that can peroxidize lipids, degrade carbohydrates and crosslink proteins. The effect of H_2O_2 on both modified and unmodified hemoglobins can be assayed by optical and magnetic techniques (Figure 18.1).

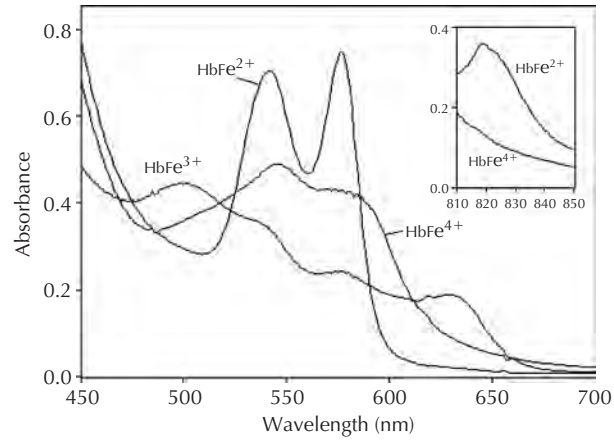

Figure 18.1 Optical spectra of hemoglobin oxidation products after reaction with hydrogen peroxide. Optical spectra of diaspirin cross-linked Hb (αα-Hb) (50 μM) in phosphate buffer, pH 7.4, before the addition of H_2O_2 ($HbFe^{2+}$). The optical spectra of metHb (50 μm) before ($HbFe^{3+}$) and 2 minutes after the addition of 100-μM H_2O_2 ($HbFe^{4+}$). Peaks, which appear at 545 and 585 nm respectively, represent the characteristic profile of the intermediate, ferryl redox state. The addition of H_2O_2 to ferrous hemoglobin generates an identical spectrum to the ferryl spectrum. The production of ferryl Hb can be confirmed by addition of 2-mM sulfide, which can generate sulfHb ($HbSFe^{2+}$) from the ferryl, indicated by the peak at 620 nm (see insert).

An additional oxidative pathway that can contribute to the toxicity of HBOCs is the formation of heme degradation products during the autoxidation of $HbFe^{2+}$, particularly under oxidative stress conditions. Recent studies have identified heme degradation by the characteristic fluorescence of these products. The heme moiety has been shown to form these degradation products during the reaction of hemoglobin with H_2O_2 (Nagababu *et al.*, 1998) and during autoxidation (Nagababu *et al.*, 2000a). Heme degradation fluorescent products were also found in red blood cells after treatment with H_2O_2 (Nagababu *et al.*, 2000b). Mechanistic studies revealed that heme degradation involves the reaction of two molecules of H_2O_2 with hemoglobin. The first molecule produces $HbFe^{4+}$, and the second molecule

undergoes a one-electron oxidation of H_2O_2 to produce $O_2^{\bullet-}$ and/or peroxide radicals ($^{\bullet}OOH$) in the heme pocket, which attack the heme, resulting in heme degradation (Nagababu *et al.*, 2000c). The formation of these degradation products by the low levels of H_2O_2 generated during autoxidation indicates the sensitivity of hemoglobin to oxidative modification and heme loss. Although there are anecdotal reports on the toxicity of free heme in a variety of disorders – such as renal hemolysis, and hemorrhage injury to the CNS – in which the hemoglobin-derived heme may promote vascular toxicity, the consequences of circulating heme and heme degradation products have been recently highlighted in a heme-oxygenase deficient child and in a knock-out mouse which lacks this enzyme, which is primarily responsible for heme breakdown. These studies have shown clearly that low levels of circulating heme can indeed produce pronounced vascular pathologies (Jeney *et al.*, 2002).

The clinical usefulness of current generation HBOCs requires that the oxygen affinity be lowered and also that the oxidative processes must be either suppressed or controlled. However, the established relationship between low oxygen affinity and increased autoxidation and possibly oxidative modification reactions suggests that these oxidative processes may actually be enhanced in some of the chemically or genetically modified HBOCs.

We have recently measured autoxidation, formation of $HbFe^{4+}$, formation of a rhombic heme (the initial step in the degradation of the heme), and fluorescent heme degradation products in a number of chemically modified hemoglobins (Nagababu *et al.*, 2002). These reactions are correlated with the functional properties of the hemoglobin, and may thus impact the safety and efficacy evaluation of HBOCs. With respect to secondary oxidative products, an interesting difference has emerged between human and bovine HBOCs. For human hemoglobins included in this study, we found an increase in the levels of heme degradation and rhombic heme when compared to bovine hemoglobins (Nagababu *et al.*, 2002).

The effects of increased levels of $HbFe^{3+}$ on the efficacy and possibly safety of HBOCs have been demonstrated in a number of animal models (Faivre *et al.*, 1998; Liberg *et al.*, 1998) and, more recently, in humans (Sprung *et al.*, 2002). The administration of HBOC-201, the human counterpart of PolyHbBv (Oxyglobin™), in surgical patients was associated with a delayed (third postoperative day) dose-dependent increase in plasma $HbFe^{3+}$ concentration (Sprung *et al.*, 2002). This is contrasted with the shorter delay, from 8 to 12 hours, in maximal $HbFe^{3+}$ concentrations after infusion of $\alpha\alpha$-diaspirin crosslinked human hemoglobin (DCLHb), the commercial analogue of $\alpha\alpha$-Hb, produced by the US Army (O'Hara *et al.*, 2001).

A comparison between $\alpha\alpha$-Hb and PolyHbBv was carried out to test whether differences in the oxidative chemistries of these two HBOCs can be demonstrated in a more physiologically relevant and simple setting. The mesenteric microvasculature and intestinal epithelium were chosen as sites to evaluate the potential of modified hemoglobins for oxidative damage because of their importance as a physiological barrier between the bloodstream and the contents of the intestinal lumen. In addition, accumulation of ROS in the intestinal mucosa, following injection of either PolyHbBv or $\alpha\alpha$-Hb in healthy rats, was monitored using a probe that only fluoresces in the presence of ROS. The association of relatively high concentrations of ROS with the blood substitute that produced the most tissue damage would support the hypothesis that hemoglobin-induced tissue damage is caused in part by the production of excess ROS. We documented that mesenteric microvascular leakage is significantly lower in animals treated with PolyHbBv compared to $\alpha\alpha$-Hb. Strengthening this finding, we also showed a lower number of degranulated mast cells present in the mesentery following perfusion with PolyHbBv compared to $\alpha\alpha$-Hb. In the intestinal mucosa, treatment with PolyHbBv resulted in more mast cell degranulation and less goblet cell activation than that produced by $\alpha\alpha$-Hb (Baldwin *et al.*, 2002).

Differences between the structures of $\alpha\alpha$-Hb and PolyHbBv may account for the differences in the intestinal toxicities observed in the treatment of healthy experimental animals. $\alpha\alpha$-Hb, an intramolecularly crosslinked tetramer, has been extensively studied *in vitro* and in animal models. *In vitro* studies on $\alpha\alpha$-Hb revealed that, besides modifying oxygen-carrying properties, subunit crosslinking with the diaspirin reagent could also affect the tendency of this hemoglobin to undergo autoxidation and subsequent oxidative side reactions such as these described earlier (Equations 18.1, 18.2). We have previously shown that PolyHbBv is more resistant to autoxidation and oxidative side reactions than $\alpha\alpha$-Hb *in vitro* (Nagababu *et al.*, 2002). The autoxidation process

is known to be a source of metHb as well as ROS. Thus, differences between the chemistries of the two hemoglobins may account for our observations regarding ROS production and tissue damage. We reason that PolyHbBv is less damaging to tissue because it causes a lower rate of production of ROS in the tissue. These studies indicated that in terms of redox-mediated cytotoxicity, PolyHbBv produces significantly less damage to microvascular networks and to the intestinal epithelium than does $\alpha\alpha$-Hb. However, there are other factors that must also be considered in the development of successful blood substitutes. For example, oxygen affinity and rate constants for oxygen uptake and release will affect rates of oxygen delivery to tissues. Recent studies on the influence of PolyHbBv on local tissue perfusion and oxygenation in acute anemia after isovolemic hemodilution in hamsters caused abnormally low tissue oxygenation and functional capillary density, while the same level of hemodilution with dextran maintained normal microvascular conditions (Tsai, 2001). Partial oxygenation (~80 per cent) at normal oxygen pressure and the insensitivity of PolyHbBv to normal allosteric modifiers of bovine hemoglobin may have contributed to the observed poor tissue oxygenation under these experimental conditions (Alayash *et al.*, 2001b).

HEMOGLOBIN AND CELL-SIGNALING PATHWAYS

Biological peroxides (i.e., H_2O_2 and $ONOO^-$) have been implicated as regulators of redox-sensitive cell-signaling pathways (Suzuki *et* al., 1997; Alayash *et al.*, 2001a). For example, reactive species have been implicated in the regulation of hematopoiesis (Sattler *et al.*, 1999). Several studies have shown that H_2O_2 regulates transcriptional and translational events in many cell types (Guyton *et al.*, 1996; Liu *et al.*, 1998; Moinova and Mulcahy, 1998). The exact targets that H_2O_2 reacts with, to either stimulate or repress a given pathway, are not known, but downstream targets include the mitogen activated protein (MAP) kinases, nuclear factor kappa B (NF-κB), and hypoxia-inducible factor (HIF) (Muller *et al.*, 1997; Zhu and Bunn, 2001). These are important components of numerous redox-sensitive signaling pathways that link extracellular stimuli to gene regulation. The effects of reactions between hemoglobin and biologically relevant peroxides in the context of cell signaling have not been explored. The concentrations of cell-free hemoglobin that can be achieved upon administration are high (in the mM range in terms of heme; Everse and Hsia, 1997), and can potentially compete with endogenous reactions that consume the peroxides mentioned. Therefore, the effects of hemoglobin on cell function may be more subtle than the oxidative damage mediated by hemoglobin, and involve the perturbation of redox-sensitive signaling pathways (Alayash *et al.*, 2001a).

HIF-1α transcription factor regulates oxygen homeostasis in cells

Hypoxia-inducible factor (HIF-1) is a transcriptional activator that functions as a global regulator of oxygen homeostasis, which facilitates both oxygen delivery and adaptation to oxygen deprivation (Semenza, 2001a). It consists of an HIF-1α subunit and an HIF-1β subunit (Wang and Semenza, 1995). HIF-1 binds to DNA in hypoxic cells but not in normoxic cells, and increases the expression of a range of target genes – including glycolytic enzymes and of angiogenic growth factors. At the mRNA level, both HIF-1α and HIF-1β are constitutively expressed. However, at the protein level HIF-1α is only found in hypoxic cells, whereas HIF-1β is constitutively expressed (Huang *et al.*, 1996). Several mechanisms responsible for HIF activation that include signal transduction via oxygen-binding hemoproteins or via the generation of $O_2^{\bullet-}/H_2O_2$, either by an NAD(P)H oxidase or the mitochondrial electron transport chain, have been suggested (Semenza, 2001b).

When bovine aortic endothelial cells (BAECs) are exposed to hypoxia ($PO_2 \sim 2$ mmHg), substantial increases in HIF-1α, tyrosine phosphorylation and HO-1 expression were seen, as measured by the Western blot technique. This is consistent with the notion that hypoxic induction of these two proteins is dependent on HIF-1α, an oxygen sensor (Semenza, 2001b). Our data suggest that cell-free hemoglobin ($\alpha\alpha$-Hb) induces changes in the levels of HIF (Figure 18.2), HO-1 and tyrosine phosphorylation, the extent of which is strongly dependent on the oxygen-carrying and redox state of this hemoglobin. We also observed a burst in the reactive oxygen species generation under 1 or 2 hours of hypoxia. Interestingly, this parallels the increases seen in hemoglobin oxidation products and the appearance of the ferryl species (Yeh and Alayash, 2004).

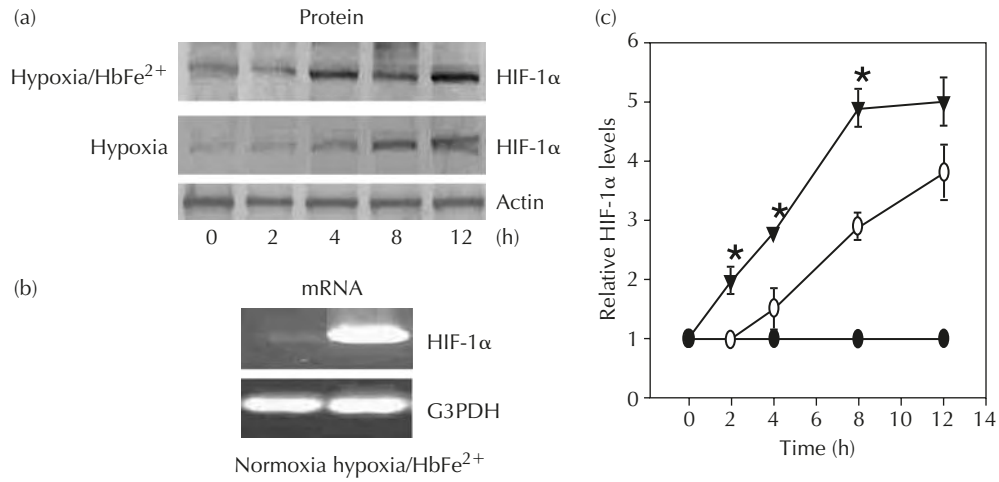

Figure 18.2 Expression of HIF-1α (protein and mRNA) in BAECs in presence or absence of $HbFe^{+2}$. BAECs were incubated under normoxic (95% air, 5% CO_2) or hypoxic (95% N_2, 5% CO_2) conditions in sealed hypoxic chamber with or without αα-Hb ($HbFe^{2+}$) (50 μM) for up to 12 hours. (a) At specific time periods, protein samples from cell lysates were analyzed by Western blot for HIF-1α expression. (b) RNA was extracted from cell samples subjected to normoxia or hypoxia with $HbFe^{2+}$ (50 μM) for 12 hours. RT-PCR was performed to amplify the cDNA products. G3PDF from the same samples were used as internal control of equal loading and normalization. (c) Densitometry was performed to quantify the corresponding bands on Western blot and plotted as a function of time. The amount of protein expression is normalized using actin as internal control. Each point represents the mean (± SD) of three experiments performed independently. Asterisks (*) signify that value is significantly greater than hypoxia alone ($P < 0.05$). Symbols represent: normoxia (closed circle), hypoxia (open circle), and hypoxia with $HbFe^{+2}$ (triangle).

Based on these experimental observations, we speculate that cell-free hemoglobin may potentially modulate cell-signaling pathways, as depicted in Figure 18.3. Both reactive oxygen and nitrogen species (ROS and RNS) can regulate transcriptional (e.g. HIF-1) and translational (e.g. heme oxygenase (HO-1)) processes. Introduction of cell-free hemoglobin and hypoxia will alter the balance between ROS and RNS. Hemoglobin reacts avidly with these species, leading to the loss of its function and its transformation into various oxidation states.

HIF-1α, a possible oxygen sensor for assessment of various oxygen-carrying blood substitutes

Generally, chemical modifications tend to stabilize the hemoglobin tetramer or its polymer in the low oxygen affinity state, a desirable property for a blood substitute. Although the aim of modification(s) is to produce a hemoglobin with oxygen characteristics close to those of red blood cells (~29 mmHg), the *P*50s exhibited by current generation HBOCs range from 6 to 52 mmHg (Riess, 2001). Some forms of chemical modification not only lower the oxygen affinity of hemoglobin to close to that of blood but also induce changes in the cooperativity, binding to carbon dioxide, chloride, and phosphates – normal allosteric modifiers of hemoglobin affinity towards oxygen. Since these various chemically modified Hbs exhibit wide range of oxygen affinities (*P*50s), allosteric sensitivities, rates of autoxidation and ROS/RNS reactivities, determination of HIF levels in endothelial cells may provide a sensitive and reliable method of measuring the tissue oxygenation and redox status under clinically relevant but well controlled cell culture conditions (Yeh and Alayash, 2003).

ANTIOXIDATIVE PROTECTIVE STRATEGIES

The recognition by many researchers that hemoglobin redox activity might limit the safety and efficacy of early generation HBOCs has prompted the design of new strategies aimed at reducing the reactivity of hemoglobin with oxidants, limiting its binding to nitric oxide, or both. These

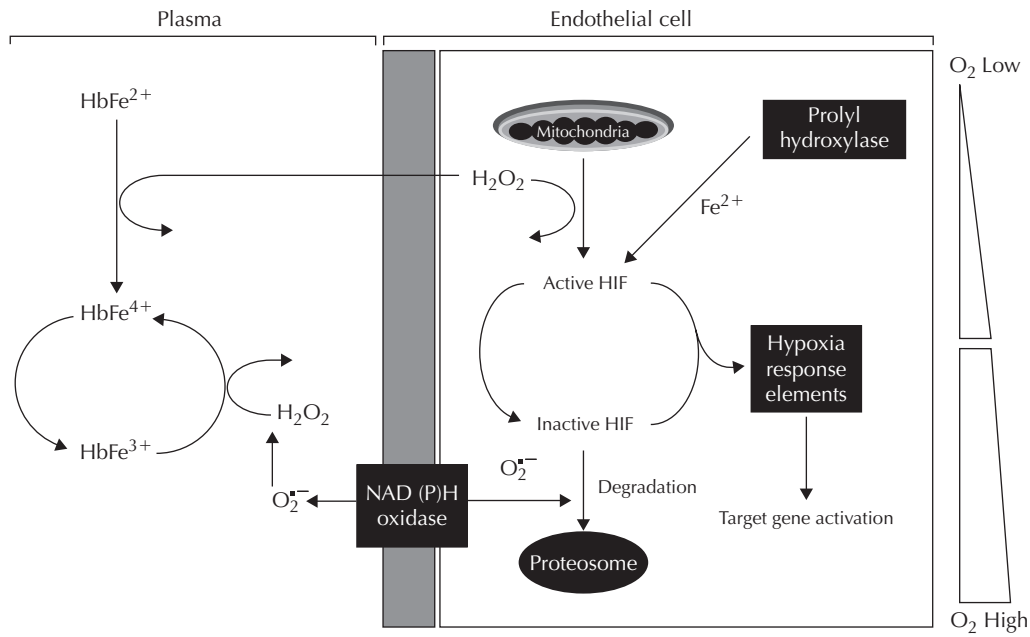

Figure 18.3 Proposed roles for HBOCs in the modulation of cell-signaling pathways. Both ROS and RNS can regulate transcription and translation processes. NADPH oxidase converts O_2 to superoxide ($O_2^{\bullet-}$) under normoxia, inactivating HIF-1α and targeting it for proteosomal degradation, thus acting as an oxygen sensor. The mitochondrial cytochrome oxidase system represents a second potential O_2 sensor. Decreased O_2 increases $O_2^{\bullet-}/H_2O_2$ and stabilizes HIF-1α. The cytoplasmic prolyl hydroxylase represents a third potential O_2-sensing mechanism by which decreased O_2 availability prevents hydroxylation of specific HIF-1α proline residues; the opposite occurs during normoxia. Introduction of hypoxia (low oxygen) and $HbFe^{+2}$ leads to changes in both hemoglobin redox states and redox-sensitive cell-signaling pathways. Hemoglobin competes with endogenous reactions that consume peroxides, resulting in increased HIF-1α expression. The reaction of hemoglobin with H_2O_2 leads to series of transitions between the ferrous ($HbFe^{2+}$), ferric ($HbFe^{3+}$) and ferryl ($HbFe^{4+}$) redox states.

strategies fall into two categories. The first is direct modification(s) of the hemoglobin molecule; including chemical and/or genetic engineering of the protein; crosslinking of red blood cell antioxidant enzymes, nitroxides, trolox (vitamin E analog), adenosine and reduced glutathione to the hemoglobin molecule; or even the use of SFH that contains red blood cell antioxidant enzymes (for review, see Alayash, 2004). The second is the indirect use of reducing agents or antioxidants. We have in recent years investigated the role of reducing agents in controlling or suppressing hemoglobin oxidative reactions *in vitro* and *in vivo*. Two biologically compatible reducing agents, selenium and ascorbic acid, were investigated in order to determine their reducing action on hemoglobin oxidation reactions *in vitro* and *in vivo*. Based on their one-electron reduction potentials in the well-established thermodynamic pecking orders of free radicals and antioxidants (Buettner, 1993), both of these reducing agents are predicted to undergo one-electron transfer reactions to hemoglobin.

Selenium significantly reduces the intestinal epithelial damage and microvasculature leakage in rats that is associated with bolus injection of HBOCs (Baldwin *et al.*, 2003, 2004). Perfusion of the tissues or feeding rats with a diet rich in selenium reduced the number of areas of leakage. In a follow-up experiment investigating the nature of its antioxidant action, selenium was found to reduce not only the rate of hemoglobin autoxidation, but also the formation of other oxidation products. Interestingly, perfusion of the microvasculature of the rats with CNmetHb produced a similar reduction in the number and areas of leakages in the microvasculature. CNmetHb is a hemoglobin in which the CN groups are tightly bound to the heme, and as such the molecule is unable to participate in redox reactions (Baldwin *et al.*, 2004).

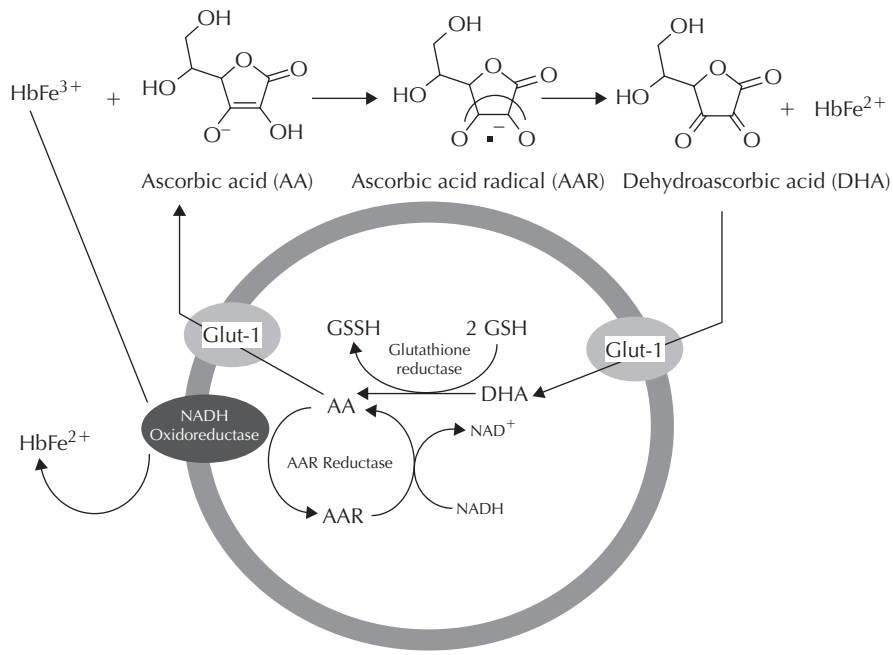

Figure 18.4 Model of ascorbic acid recycling by erythrocytes and its potential contribution to redox control of cell-free hemoglobin oxidation. Extracellular oxidants, such as metHb, oxidize intracellular ascorbic acid (vitamin C) (through the action of a transmembrane oxidoreductase) to dehydroascorbic acid (DHA), which enters the red cell via the glucose transporter. Intracellular DHA is converted back to ascorbic acid via GSH- or NADH-dependent reduction, which then leaves the cell via the glucose transporter.

The reducing properties of ascorbic acid (vitamin C) are well established in a number of biological systems. We have shown that ascorbic acid can specifically reduce ferryl heme formation of myoglobin (Mb), and reduces apoptosis (programmed cell death) in endothelial cells exposed to H_2O_2 and Mb (D'Agnillo and Alayash, 2002). Using electron paramagnetic resonance (EPR) in a model of 20 per cent exchange transfusion, we recently detected several paramagnetic oxidation species of cell-free hemoglobin – including the ferryl radical in rabbits (Dunne *et al.*, unpublished data). The role of the organism's own protective mechanisms in controlling oxidation of infused free hemoglobin in plasma (May *et al.*, 2004) was specifically explored in our animal model. We showed that ascorbic acid, which is known to recycle between the red blood cells and plasma, decreases free metHb in plasma (Figure 18.4). These findings offer a number of important implications for the clinical use of HBOCs. First, the co-administration of reducing agents, such as selenium or ascorbic acid with hemoglobin may offer a simple strategy to control or reduce hemoglobin-mediated toxicity. Secondly, in the case of ascorbic acid the hematocrit should not be allowed to drop too low otherwise metHb reduction will be compromised. Finally, the use of appropriate and relevant animal models to study the toxicity of hemoglobin-based blood substitutes is highly desirable. There appear to be animal- and tissue-specific expressions of the gene for L-gluconolactone oxidase, the enzyme responsible for the biosynthesis of ascorbic acid. Thus it follows that animals' reductive as well as antioxidative mechanisms should be taken into account when these studies are designed.

SUMMARY

Extensive studies carried out in our laboratory and in the laboratories of others clearly show that the type of chemistry introduced to stabilize and functionally modify hemoglobins can indeed determine the vulnerability of hemoglobin to undergoing oxidative self-destructive side reactions. Some forms of chemical modifications not only modify key amino acids on the surface of the protein (e.g. amino acids involved in Bohr and chloride effects and carbon dioxide binding), but can also have far-reaching consequences

including the distorting of heme geometry (rhombic heme) and loss of its integrity. At the cellular levels, differences in the rates of autoxidation and oxidative side reactions not only determine the ability of hemoglobin to induce injury, but also can determine its ability to deliver oxygen. Different HBOCs can differentially modulate key cell-signaling pathways and other important physiological mediators. We have demonstrated that hemoglobin 'oxygen carrier' does indeed 'cross-talk' with HIF 'oxygen sensor' in endothelial cells subjected to normoxia, and more so in hypoxia. Changes in the expression of HIF and other important signaling proteins are clearly dependent on the oxygen-carrying and redox state of hemoglobin. Utilization of animal antioxidant defense mechanisms, including the contribution of red blood cells, should be considered in the design of animal studies and possibly in humans. The co-administration of a well-known reducing agent or antioxidant may provide an even simpler protective strategy.

EDITOR'S SUMMARY

The oxidation-reduction (redox) reactions between ferrous and ferric oxygen can generate electrons that can participate in an array of oxidative reactions, some of which could be toxic. Although the direct connection between these reactions and hemoglobin toxicity has not been proven conclusively, research has focused on these mechanisms and ways to alleviate the effects. The redox reactions, in which cell-free hemoglobin can participate, can generate biological peroxides, i.e., hydrogen peroxide (H_2O_2), lipid peroxide (LOOH), and peroxynitrite ($ONOO^-$), which can participate in multiple biochemical reactions at the cellular, tissue and organ levels.

These peroxides have also been implicated as regulators of redox-sensitive cell-signaling pathways, offering another way in which cell-free hemoglobin might interfere with normal homeostasis. The effects of reactions between hemoglobin and biologically relevant peroxides may be more subtle than oxidative damage, and may thus involve perturbation of redox-sensitive signaling pathways.

This chapter has presented a brief outline of the potential role of cell-free hemoglobin in oxidative and cell-signaling pathways and the implications of these reactions on the safety and efficacy of hemoglobin-based oxygen carriers. Also included are some molecular interventions that can potentially be employed, directly or indirectly, to overcome the toxic side reactions of hemoglobin.

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Chapter 19

Pro-Oxidant Activity of Hemoglobin and Endothelial Cytotoxicity

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INTRODUCTION

Several promising hemoglobin-based oxygen carriers (HBOCs) are being developed. Potential applications include resuscitation from hypovolemic shock, preoperative hemodilution for elective surgery, treatment of ischemic disease, and others. Over the past two decades, several chemical and recombinant approaches have been used to impart certain modifications to the hemoglobin molecule. These modifications have been successful in countering several of the early obstacles in the search for an acellular hemoglobin-based oxygen-carrying solution. Despite these improvements, lingering efficacy and safety issues have slowed progress in the field. One commonly cited limitation is the vasopressor effect reported with most HBOCs (Alayash, 1999; Winslow, 2000). The mechanism, although still debated, is believed to involve the hemoglobin-mediated binding of the endogenous vasodilator molecule, nitric oxide (NO) (Rohlfs *et al.*, 1998; Alayash, 1999). Another significant consideration, although less appreciated, is the inherent pro-oxidant activity of hemoglobin and its deleterious consequences. More recent protein development strategies have specifically addressed some of these lingering issues. For example, genetically modified hemoglobins have been developed with restricted nitric oxide binding, and antioxidant enzymes have been chemically attached to hemoglobin to limit its pro-oxidant activity (D'Agnillo and Chang, 1998a; Doherty *et al.*, 1998).

A clear and complete understanding of how HBOCs interact with the vascular system has yet to be established. Vascular endothelium likely plays a key role in determining the overall response to HBOCs. Our laboratory and others have described a series of oxidative mechanisms by which cell-free hemoglobin and HBOCs can damage cultured vascular endothelial cells. However, despite extensive evidence supporting the harmful nature of free hemoglobin in the vasculature, it is not yet clear whether the specific oxidative processes or toxicities observed *in vitro* occur following the administration of HBOCs *in vivo*. This chapter will focus on the pro-oxidant activities of hemoglobin, with specific emphasis on the redox cycling reactions involving ferryl hemoglobin. An overview of *in vitro* and *in vivo* studies that have focused on the interaction of cell-free hemoglobin and HBOCs with the microvasculature will also be presented.

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PRO-OXIDANT ACTIVITY OF HEMOGLOBIN

Heme moiety of hemoglobin

Hemoglobin is a tetrameric protein composed of two alpha (α) and two beta (β) chains. Each globin chain contains an iron-bearing heme prosthetic group. The porphyrin ring structure of heme contains four pyrrole rings. Iron is bound to the four nitrogen atoms of the ring and to the imidazole nitrogen of the proximal histidine at positions 87 and 92 in the alpha and beta chains, respectively. Heme containing iron in the ferrous (Fe^{2+}) state is capable of sharing an electron to allow the reversible binding of oxygen. However, the higher oxidation states of heme iron, such as the ferric (Fe^{3+}) or ferryl (Fe^{4+}) forms, cannot reversibly bind molecular oxygen. Inside the red blood cell, a number of different factors contribute to the maintenance of the functional ferrous heme form. The positioning of heme in a region surrounded by non-polar residues excludes water molecules, thus protecting ferrous iron from oxidation. In addition, the tetrameric conformation of hemoglobin also assists in protecting the ferrous form, since $\alpha\beta$ dimers and isolated globin chains are more susceptible to oxidation. Red blood cell antioxidant defense systems and methemoglobin reducing systems also play a critical role in maintaining the ferrous form of hemoglobin. Purified hemoglobin, used in the preparation of HBOCs, is therefore highly susceptible to interaction with intravascular oxidants and/or oxidants generated from its autoxidation.

Redox cycling reactions

Significant attention has focused on the interaction of hydrogen peroxide (H_2O_2) and organic peroxides (ROOH) with hemoglobin (Giulivi and Davies, 1994; D'Agnillo and Alayash, 2000a; Alayash *et al.*, 2001). Both ferrous hemoglobin (oxy- and deoxy- forms) and ferric hemoglobin can react with H_2O_2 to produce ferryl Hb ($HbFe^{IV} = O$) (Equations 1–5 in Table 19.1). In the case of ferric hemoglobin, this reaction also generates a protein-based radical ($^{\bullet}HbFe^{IV} = O$) that can be detected by electron paramagnetic spectroscopy (Equation 3 in Table 19.1). Ferryl Hb decays to ferric Hb through a disproportionation reaction with ferrous Hb, via auto-reduction, or following oxidation of other substrates. With ferrous Hb as the starting species, this process can also be accompanied by the formation of fluorescent heme degradation products via a superoxide-driven mechanism (Equation 4 in Table 19.1) (Nagababu and Rifkind, 2000). The redox potential of ferryl Hb ($\sim$1.6 V) indicates that its oxidizing capability is close to that of the potent hydroxyl radical ($^{\bullet}OH$, 2.3 V). Both the radical and non-radical forms of ferryl Hb are capable of oxidizing lipids and other biological molecules. In particular, the ferryl species can generate stable F_2-isoprostane compounds through the peroxidation of arachidonic acid. F_2-isoprostanes are potent vasoconstrictors, and are thus particularly interesting given the vasopressor effects associated with most HBOCs (Reeder *et al.*, 2002).

With the bolus addition of low or equimolar H_2O_2 to ferrous or ferric Hb, H_2O_2 is rapidly

Table 19.1 Redox reactions of hemoglobin

Redox mechanism	Reaction equation	
H_2O_2-Driven Redox Cycling	$HbFe^{2+}O_2 + H_2O_2 \rightarrow HbFe^{4+} = O + H_2O + O_2$	(Eq. 1)
	$HbFe^{2+} + H_2O_2 \rightarrow HbFe^{4+} = O + H_2O$	(Eq. 2)
	$HbFe^{3+} + H_2O_2 \rightarrow {}^{\bullet}HbFe^{4+} = O + H_2O$	(Eq. 3)
	$HbFe^{4+} = O + H_2O_2 \rightarrow HbFe^{3+} + H_2O + O_2^{\bullet-}$	(Eq. 4)
	$^{\bullet}HbFe^{4+} = O + H_2O_2 \rightarrow HbFe^{3+} + H_2O + O_2$	(Eq. 5)
Autoxidation	$HbFe^{2+}O_2 \rightarrow HbFe^{3+} + O_2^{\bullet-}$	(Eq. 6)
Hydroxyl Radical Formation	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$	(Eq. 7)
	$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$	(Eq. 8)
	$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$	(Eq. 9)
(Net reaction)	$H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + {}^{\bullet}OH + OH^-$	(Eq. 10)

consumed and ferric Hb gradually accumulates as ferryl Hb decays (Equations 1–3 in Table 19.1). It can also be interpreted from these equations that low continuous fluxes of H_2O_2 can generate ferryl Hb and, at the same time, sustain the ferryl–ferric transition by reacting with ferric Hb. The redox cycling of ferryl-ferric species can be achieved experimentally by incubating Hb with the H_2O_2-generating glucose–glucose oxidase system (Giulivi and Davies, 1994; D'Agnillo and Alayash, 2001). Under these conditions, 'regenerated' ferryl Hb is once again available to propagate oxidative damage in biological systems. Using enzymatic systems to generate low steady state concentrations of H_2O_2 is also considered a more physiologically relevant approach to study the redox activity of Hb in experimental settings. For example, the glucose oxidase system may be useful in mimicking the mode of extracellular oxidant production from *in vivo* sources such as xanthine oxidase and NADPH oxidase. Both these enzymes may play prominent roles in driving the oxidative reactions of Hb in the vasculature (Granger and Korthius, 1995; Cai and Harrison, 2000).

It is important to recognize that HBOCs may exhibit differences in their susceptibility to undergoing redox reactions (Osawa *et al.*, 1993; Cashon and Alayash, 1995). In some cases, these differences lie in the nature of the chemical modification. For example, alpha crosslinked Hb ($\alpha\alpha$-Hb) generates more ferryl Hb upon H_2O_2 exposure than native Hb, while $\beta\beta$ crosslinked Hb exhibits less ferryl radical upon similar oxidant challenge (Cashon and Alayash, 1995). Gluteraldehyde modification has also been shown to affect oxidative susceptibility (Alayash, 1995). In other cases, the purity of the starting Hb material used to produce a particular HBOC or the inclusion of antioxidants in certain formulations may limit the pro-oxidant activity of the final HBOC preparation (Privalle *et al.*, 2000).

Other pro-oxidant mechanisms of hemoglobin

Oxyhemoglobin undergoes slow spontaneous autoxidation in the red blood cell (Equation 6 in Table 19.1). About 1–3 per cent of hemoglobin contained in red blood cells is present as non-oxygen binding ferric Hb (i.e., methemoglobin). This autoxidation also generates superoxide anion ($O_2^{\bullet-}$), the one-electron reduction product of molecular oxygen. While $O_2^{\bullet-}$ itself is not considered a major cytotoxic species, it participates in the formation of secondary, more toxic radical species. This occurs in part through its dismutation to H_2O_2, which occurs spontaneously or via the enzyme superoxide dismutase (SOD) (Equation 7 in Table 19.1). Uncontrolled autoxidation of Hb in the circulation has been a source of concern with HBOCs (Lee *et al.*, 1995; Linberg *et al.*, 1998; Alayash, 1999). Potential conversion to large quantities of methemoglobin not only eliminates the HBOC's usefulness as an oxygen carrier but also subjects the microcirculation to potential harmful effects of less stable ferric Hb. The presence of reductants in plasma such as ascorbate may help limit the autoxidation process (Faivre *et al.*, 1994). The autoxidation kinetics of a given HBOC can also be affected by the nature of its chemical modification and/or by the presence of residual red blood cell proteins (Yang and Olsen, 1989; D'Agnillo and Alayash, 2000a).

Hemoglobin can also release free iron or heme, especially when free in the circulation. In some cases, the breakdown products of circulating Hb can overwhelm natural iron- and heme-binding defense mechanisms such as transferrin, haptoglobin, and hemopexin. In the presence of free iron, $O_2^{\bullet-}$ and H_2O_2 rapidly react to produce hydroxyl radical ($^{\bullet}OH$) via the iron-catalyzed Haber–Weiss reaction or superoxide-driven Fenton reaction (Equation 10 in Table 19.1). This highly reactive species can damage lipids, proteins and nucleic acids within a small radius of its site of production. In this scheme, $O_2^{\bullet-}$ plays a dual role by both reducing ferric iron and acting as a source of H_2O_2 (Equations 7 and 8 in Table 19.1). Excessive oxidative damage to Hb can lead to the release of iron from heme and hydroxyl radical generation (Gutteridge, 1986). For example, polymerized bovine Hb treated with millimolar amounts of H_2O_2 released iron and generated hydroxyl radical as detected by salicylate hydroxylation products (D'Agnillo and Chang, 1998b). The significance of this specific pathway *in vivo*, however, has been debated because of the high oxidant concentrations required to release heme/iron from Hb.

Significant attention has also focused on the reaction of Hb with nitric oxide, which is widely considered to be responsible for the vasopressor effect observed in preclinical and clinical studies with most HBOCs (Alayash, 1999). Oxyhemoglobin reacts very rapidly with nitric oxide at the heme site, generating ferric Hb and nitrate. The reaction of nitric oxide with ferric Hb is characterized by the initial rapid formation of a

nitric oxide–ferric heme adduct, followed by a slower process whereby nitric oxide reduces heme to the ferrous state, forming a nitric oxide–ferrous heme complex. Compared to native Hb, αα-Hb displays a faster rate of nitric oxide–ferric heme formation and a faster nitric oxide reduction to ferrous heme (D'Agnillo and Alayash, 2000a). Clearly, this implies that chemical modifications affecting the orientation of the heme pocket can alter nitric oxide-binding kinetics. This has generated interest in genetically modifying the heme pocket to produce HBOCs that may be less vasoactive (Doherty *et al.*, 1998). Nitric oxide also reacts rapidly with $O_2^{\bullet -}$ to generate peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996). This highly oxidizing species may play a role in several oxidative stress conditions, such as ischemia-reperfusion injury and septic shock. Peroxynitrite is a more potent oxidizer of Hb than H_2O_2. The reaction of ferrous Hb with $ONOO^-$ apparently proceeds via a direct one-electron oxidation to generate ferric Hb (Alayash *et al.*, 1998). Some reports indicate that nitric oxide may serve an antioxidant role by reducing ferryl Hb (Gorbunov *et al.*, 1995).

HEMOGLOBIN-MEDIATED ENDOTHELIAL CYTOTOXICITY *IN VITRO*

Heme- and ferryl Hb-induced cytotoxicity

Endothelial cells are highly susceptible to cytotoxic effects of heme released by Hb (for review, see Balla *et al.*, 2003). Ferric Hb was shown to release heme more readily than its ferrous counterpart, and induced greater endothelial cytotoxicity (Balla *et al.*, 1993). Activated polymorphonuclear cells promoted this effect by oxidizing ferrous Hb to methemoglobin (Balla *et al.*, 1991). Endothelial cells exposed to Hb showed an increased expression of heme oxygenase-1 (HO-1), the enzyme responsible for heme degradation, and an increased susceptibility to H_2O_2 cytotoxicity. In the latter studies, ferritin, a main intracellular iron storage protein, also showed increased expression. Enhanced autoxidation of αα-Hb compared to native Hb correlated with a higher increase in endothelial HO-1 expression (Motterlini *et al.*, 1995). Partial protection by desferrioxamine indicated the possible involvement of a free iron-mediated mechanism. It was recently shown that an HO-1 deficient-child exhibited increased hemolysis and endothelial injury (Kawashima *et al.*, 2002). Knockout mice deficient in HO-1 are also markedly more sensitive to heme damage (Nath *et al.*, 2000).

A number of studies have examined the cytotoxic effects of ferryl Hb in endothelial cell culture. In a hypoxia/reoxygenation endothelial model, the ferryl form of αα-Hb was detected during the reoxygenation stage, and ferryl formation closely paralleled cellular lipid peroxidation (McLeod and Alayash, 1999; D'Agnillo *et al.*, 2000). H_2O_2 released from hypoxia-reoxygenated endothelial cells drove the ferryl formation. Endothelial cells treated with αα-Hb or native Hb and bolus amounts of H_2O_2 sustained necrotic-type injury (D'Agnillo and Alayash, 2000b). A direct correlation between ferryl Hb formation by bolus H_2O_2 and the depletion of endothelial cell glutathione (GSH) was also demonstrated. In the latter experiments, the cytotoxicity component directly attributable to Hb redox reactions was difficult to distinguish because the bolus addition of millimolar amounts of H_2O_2 alone caused considerable cytotoxicity. In fact, native Hb or αα-Hb, through its rapid reaction with H_2O_2, partially attenuated bolus H_2O_2-mediated apoptotic endothelial injury.

H_2O_2 is continuously produced and remains in a quasi-steady state *in vivo*. The steady state H_2O_2 concentration in human plasma has been reported to be 10–100 nM, while some have reported levels as high as 5–35 μM depending on the type of methodology (Yamamoto *et al.*, 1987; Halliwell *et al.*, 2000). More recent cell culture studies have employed the glucose oxidase system to enzymatically generate low steady state concentrations of H_2O_2 ranging from 1 to 10 μM (Antunes and Cadenas, 2001; D'Agnillo and Alayash, 2001). In this experimental setting, low continuous fluxes of H_2O_2 sustain the redox cycling of ferric–ferryl Hb and allow the effects of ferryl–ferric redox cycling to be examined without the confounding effects of bolus oxidant addition. Using this experimental approach in bovine endothelial cell culture, it was shown that ferryl–ferric redox cycling of cell-free Hb and αα-Hb caused $G_{2/M}$ cell cycle arrest and apoptotic cell death (Figure 19.1; D'Agnillo and Alayash, 2001). Similar findings have been observed with different chemically polymerized Hbs (unpublished results). In the presence of glucose oxidase, both ferrous and ferric forms of αα-Hb produced growth arrest and apoptosis. This effect was inhibited when the heme site was blocked with cyanide (CNmet-αα-Hb) (Figure 19.2). Other experiments also revealed a close correlation

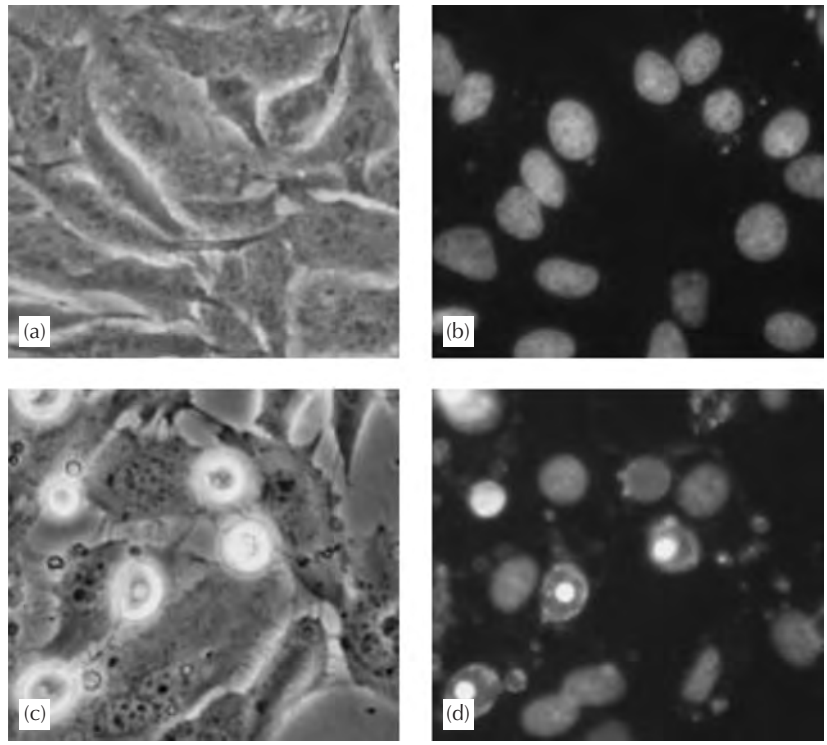

Figure 19.1 Redox cycling of αα-Hb causes endothelial cell cycle arrest and apoptosis. Bovine aortic endothelial cells were treated with (a, b) serum-free medium alone, or (c, d) medium containing 50 μM αα-Hb and H_2O_2-generating glucose oxidase. After 16 hours, cells were stained with Hoechst 33342, Alexa Fluor 488 annexin V conjugate, and propidium iodide (PI), and visualized by phase contrast and immunofluorescence microscopy (for detailed description see D'Agnillo, 2004). Hoechst 33342, a cell permeant blue fluorescent DNA dye, was used to visualize nuclear morphology. Alexa Fluor 488-annexin V conjugate was used to detect phosphatidylserine on the outer leaflet of the plasma membrane, an early apoptotic marker. PI, a red fluorescent nucleic acid dye, enters and stains cells with decreased plasma membrane integrity. Control cultures showed an intact monolayer with typical pale blue nuclei staining by Hoechst 33342 and no annexin V or PI staining (a, b). Treatment with αα-Hb and glucose oxidase caused cell rounding, detachment, and condensation of nuclei stained brightly by Hoechst coupled with annexin V plasma membrane staining (green), indicative of apoptosis (c, d). Glucose oxidase or αα-Hb alone did not produce these morphological changes (not shown). See color plate 3.

between the redox cycling characteristics of Hbs and HBOCs and the extent of cellular injury. Different endothelial cell types such as human coronary artery cells were also shown to undergo this form of ferryl-mediated injury. The underlying oxidative processes that lead to growth arrest and apoptosis are not yet known. However, the protective effects of catalase, a scavenger of H_2O_2, and ascorbate, a known reductant of the ferryl species, as well as the increased susceptibility of GSH-depleted endothelial cells to ferryl cytotoxicity clearly implicate a role for oxidative stress in this injury process (D'Agnillo and Alayash, 2001, 2002).

Some investigators have shown that oxyhemoglobin alone activates endothelial cell apoptosis (Ogihara *et al.*, 1999). This finding contrasts with the observations from our laboratory indicating that purified Hb and HBOC preparations are not, by themselves, pro-apoptotic in endothelial cell culture (D'Agnillo and Alayash, 2000c). The studies in question employed a commercial Hb with an undetermined purity profile, raising the possibility that contaminants contributed to the observed apoptosis. This example illustrates the importance of carefully considering the source and characterization profile of a particular Hb or HBOC when establishing their effects.

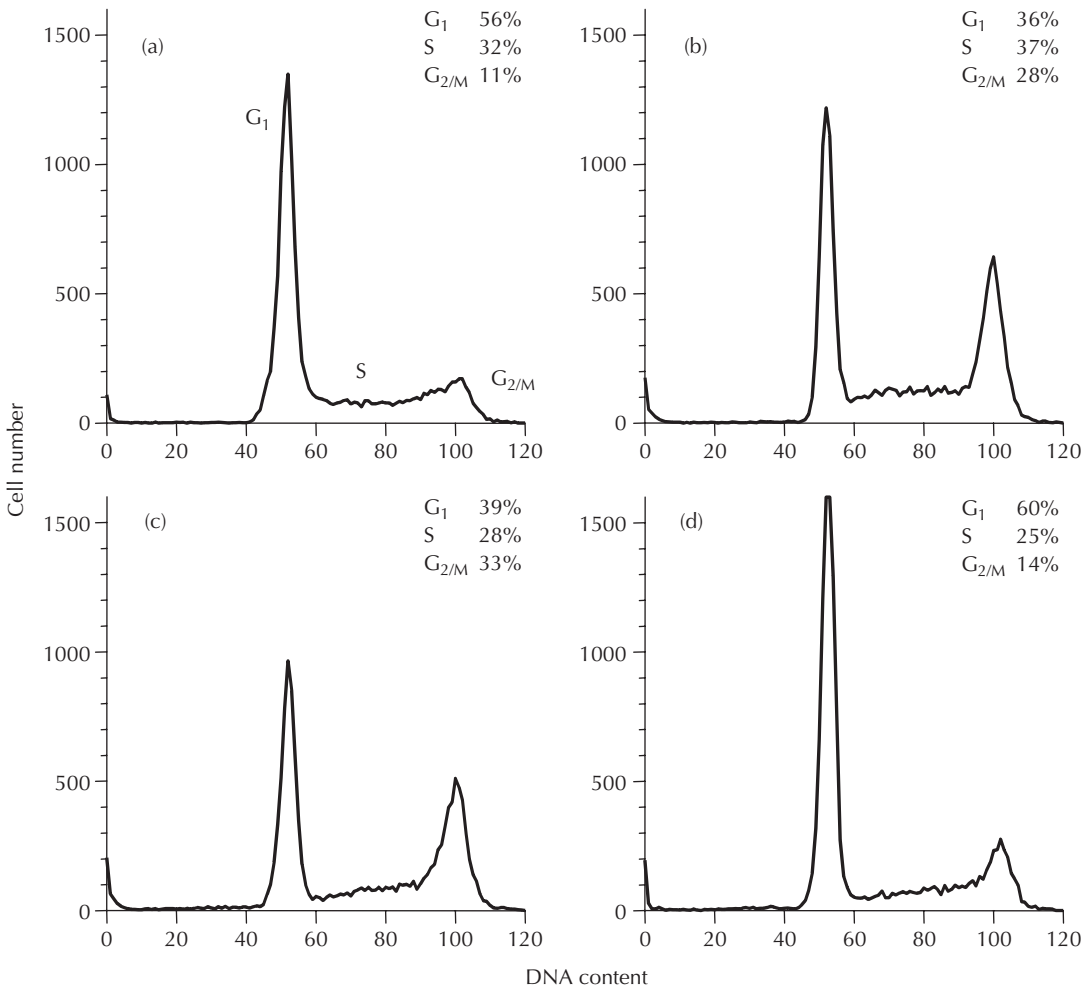

Figure 19.2 Ferrous and ferric αα-Hb, but not the CN-Met form, induce endothelial cell cycle arrest. Bovine aortic endothelial cells were treated with (a) serum-free medium alone, or medium containing 50 μM αα-Hb in the (b) ferrous, (c) ferric or (d) CN-met form and H_2O_2-generating glucose oxidase. After 9 hours, adherent and non-adherent cells were pooled and fixed in 70 per cent ethanol. DNA content was determined by flow cytometry following RNase digestion and PI staining. DNA content histograms with calculated fractions of G_1, S, and $G_{2/M}$ from a representative experiment are shown. Both ferrous and ferric forms of αα-Hb, but not the CN-Met form, led to $G_{2/M}$ cell cycle arrest as indicated by the accumulation of $G_{2/M}$ cells.

Redox cycling of hemoglobin enhances lipopolysaccharide-induced cytotoxicity

Several animal studies indicate that cell-free Hb or HBOCs may exacerbate the pathophysiology of sepsis or endotoxemia (White *et al.*, 1986; Krishnamurti *et al.*, 1997; Su *et al.*, 1997, 1999). A synergistic effect between Hb and lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria and the primary mediator of gram-negative sepsis, has been implicated. One proposed mechanism suggests that Hb enhances the biological activity of LPS by promoting the disaggregation of LPS complexes (Kaca *et al.*, 1994). Some forms of LPS can also directly oxidize Hb (Kaca *et al.*, 1995).

Recent *in vitro* findings support a possible mechanism whereby the redox cycling of Hb, and not its direct interaction with LPS, contributes to Hb-mediated enhancement of LPS-related pathophysiology (D'Agnillo, 2004). The latter studies found that co-incubation of bovine Hb, human Hb, or αα-Hb with LPS did not promote endothelial apoptosis in the absence of serum, nor alter the extent of LPS-induced apoptosis in the presence of serum. In contrast, the redox cycling of these Hbs, driven by a low

level of oxidative stress, significantly exacerbated LPS-induced apoptosis. It is interesting to speculate on the relationship between the latter observations and the synergistic toxicity of cell-free Hb and LPS observed in animals.

Sepsis and other pro-inflammatory conditions are generally accompanied by oxidative stress and the release of inflammatory mediators. Xanthine oxidase and phagocyte NADPH oxidase, two enzymatic sources for $O_2^{\bullet -}$ and H_2O_2, have been implicated in the overproduction reactive oxygen species in sepsis and endotoxemia (Brandes *et al.*, 1999; Sato *et al.*, 2002). Increased lipid peroxidation and marked depletion of antioxidants such as ascorbate, vitamin E and GSH have been observed in clinical and experimental sepsis (Hung, 2000; Wiesel *et al.*, 2000; Lyons *et al.*, 2001; MacDonald *et al.*, 2003; Motoyama *et al.*, 2003). It is conceivable that this oxidative environment could facilitate the pro-oxidant activity of Hb and contribute to sepsis pathophysiology. The ferryl species was indirectly detected in the peritoneal fluid of animals subjected to experimental *E. coli* peritonitis (Yoo *et al.*, 1999). The potential role of redox active Hb in sepsis or endotoxemia requires further investigation.

PRO-OXIDANT ACTIVITY OF HEMOGLOBIN AND VASCULAR TOXICITY *IN VIVO*

Vascular endothelial injury and dysfunction often accompany the intravascular hemolysis observed with cerebral hemorrhage, infections, hemodialysis, and certain red cell disorders. This attests to the damaging effects of free Hb in the circulation (for review, see Alayash *et al.*, 2001). However, direct evidence linking the pro-oxidant activity of HBOCs to oxidative toxicity *in vivo* has not been fully established. Studying the pro-oxidant activities of Hb *in vivo* has been complicated by the lack of adequate methodologies to measure these events directly. The measurement of heme to protein crosslinked Hb species could serve as a useful *in vivo* indicator of ferryl-mediated events (Reeder *et al.*, 2002). Heme to protein crosslinked Hb species, which contain a covalent bond between heme and the globin component, are specifically generated via ferryl redox reactions. Increased levels of these heme to protein crosslinked Hb species were found in the cerebrospinal fluid of patients following subarachnoid hemorrhage, suggesting a possible role for ferryl Hb in the delayed vasospasm associated with subarachnoid hemorrhage (Reeder *et al.*, 2002). The crosslinked heme to protein form of myoglobin (Mb) has also been measured in the urine of patients with rhabdomyolysis (Holt *et al.*, 1999). Increased lipid peroxidation with the concurrent production of F_2-isoprostanes occurs in animals with glycerol-induced rhabdomyolysis and in patients with rhabdomyolysis (Moore *et al.*, 1998). These effects were caused by lipid peroxide- or H_2O_2-driven formation of ferryl Hb in kidneys, and not to free heme or iron-mediated events. The improved ability to measure more stable oxidative markers such as specific crosslinked heme to protein compounds or F_2-isoprostane lipid peroxidation products will help clarify the link between the oxidative nature of Hb and vascular injury.

Peroxidative-like injury to swine liver and kidney was demonstrated following exchange transfusion with $\alpha\alpha$-Hb (Smith *et al.*, 1990). Exchange transfusion with modified bovine Hb increased lipid peroxidation in rat heart, liver and plasma (Simoni *et al.*, 1995). Another study found that desferrioxamine reversed the vasopressor effect of $\alpha\alpha$-Hb in rabbit hearts, presumably by inhibiting iron-mediated free radical processes (Motterlini and MacDonald, 1993). However, this does not definitely implicate free iron since desferrioxamine possesses a number of other functions, including the ability to reduce ferryl Hb. The administration of $\alpha\alpha$-Hb in rats induced hepatic HO-1, suggesting a protective response triggered to counter potential heme-related toxicity (Przybocki *et al.*, 1998). Resuscitation of hemorrhaged dogs with cell-free Hb increased the formation of salicylate hydroxylation products, indicative of $^{\bullet}OH$ formation (Biro *et al.*, 1995). Pre-clinical studies have shown that administration of Baxter's version of $\alpha\alpha$-Hb (DCLHb™) induces necrotic lesions in animal hearts (see Chapter 23). These lesions were localized to highly vascularized regions of the heart and believed to involve the reaction of Hb with nitric oxide (Burhop *et al.*, 2004).

The administration of $\alpha\alpha$-Hb in rats increased albumin leakage through the mesenteric microvasculature (Baldwin *et al.*, 2003). Similar experiments with polymerized bovine Hb (PolyBvHb) showed significantly reduced leakage compared to $\alpha\alpha$-Hb (Baldwin *et al.*, 2003). The reduced leakage was linked to the decreased pro-oxidant activity of polymerized bovine Hb compared to $\alpha\alpha$-Hb. Protective effects of selenium, an antioxidant, in this system and

others have also been demonstrated (Simoni *et al.*, 1995; Baldwin *et al.*, 2004). Another antioxidant approach involves crosslinking the antioxidant enzymes SOD and catalase to Hb (PolyHb-SOD-CAT) (D'Agnillo and Chang, 1998a). This approach has shown beneficial effects compared to PolyHb alone in ischemia-reperfusion (I-R) injury models (Razack *et al.*, 1997; Powanda *et al.*, 2002). I-R injury is caused by oxidants produced following the return of oxygen to ischemic tissues. Under these oxidative stress conditions, HBOCs could further promote and exacerbate oxidant tissue damage. In this context, a desirable oxygen carrier may be one that delivers oxygen effectively, and at the same time prevents the increase in oxidants and/or ameliorates pre-existing oxidative stress. An important clinical consideration, given the pro-oxidant nature of Hb, is the antioxidant status of individuals receiving HBOCs. Individuals with diabetes, hypertension, myocardial infarction, acute ischemic stroke, hemorrhagic shock and sepsis suffer from compromised vasculatures and poor antioxidant status (Robinson *et al.*, 1992; Palace *et al.*, 1999; Wernerman *et al.*, 1999; Dhalla *et al.*, 2000). Under these circumstances, individuals may be more susceptible to the adverse effects of HBOCs.

SUMMARY

Several promising HBOCs are currently being developed. Understanding how these products interact with the vascular system is an important factor when considering their safety and efficacy. The inherent pro-oxidant activity of cell-free Hb is one potential mechanism that can promote certain adverse reactions. Growing recognition that Hb redox chemistry may limit the safety and efficacy of first-generation HBOCs has already prompted the design of strategies aimed at reducing the pro-oxidant activity of Hb and limiting the binding of nitric oxide. A direct link, however, between the *in vitro* cytotoxicity of Hb and HBOCs and the effects of HBOCs *in vivo* has not been fully established. Studying these mechanisms *in vivo* may now be more feasible by measuring stable oxidative markers such as specific ferryl-generated Hb species and F_2-isoprotane peroxidation products.

EDITOR'S SUMMARY

Vascular endothelium is the interface to all contents of the circulation. Endothelial cells can be studied both *in vitro* and *in vivo*, and represent a powerful system to evaluate the cellular and biochemical reactions that occur when cell-free oxygen carriers are introduced into the body. One potential concern is the production of reactive oxygen species. Such species are powerful oxidizing agents, and can lead to detrimental intracellular effects.

Cytotoxicity, including apoptosis (programmed cell death), can result from the production of reactive oxygen species during the redox cycling of iron between the Fe^{2+}, Fe^{3+} and Fe^{4+} forms. At present this toxicity remains speculative, because there is no proven link between these *ex vivo* effects and any clinical toxicity. However, as products are tested in an ever widening circle of patients with a larger range of underlying disease, it is possible that some products may be found to be more toxic in certain classes of patients than others.

A confounding factor in this research is that not all hemoglobin-based products are equivalent in key properties, such as the propensity to oxidation, release of heme from the protein, oxygen affinity, molecular size and rate of extravasation. Therefore, care must be taken not to generalize the results from any one product to all products.

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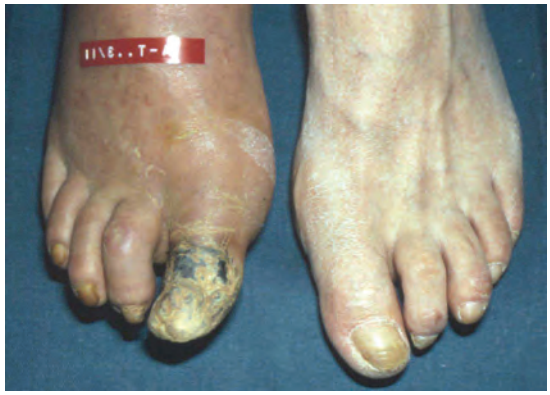

Plate 1 A patient with severe chronic critical leg ischemia (CCLI) in his right foot. See Fig. 17.1.

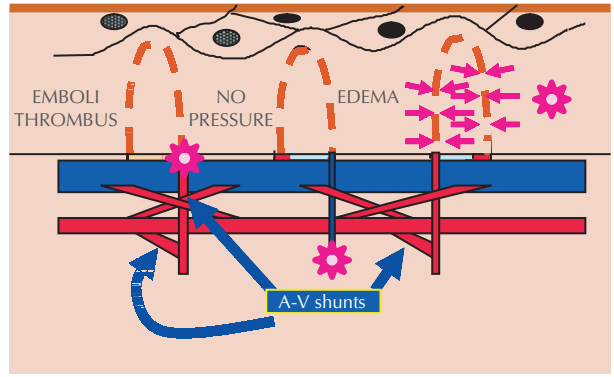

Plate 2 Schematic drawing of the skin microcirculation in an ischemic region. There are three possible reasons for blood cells not entering the nutritional capillaries: (1) microthrombo-emboli; (2) too low an inflow pressure to open the precapillary arterioles; and (3) interstitial edema compressing the arteries. Plasma can usually enter the capillaries. See Fig. 17.2.

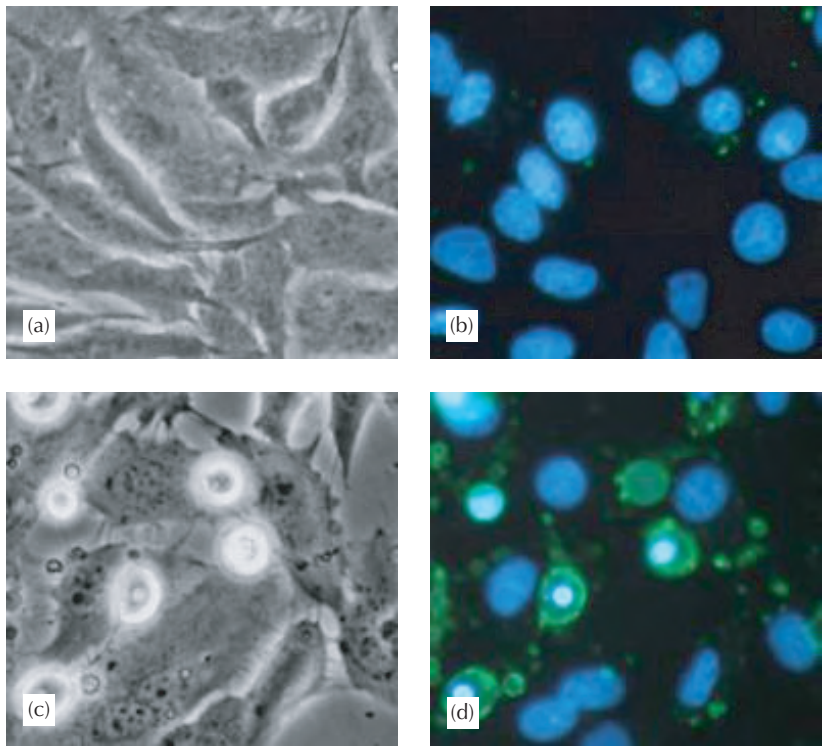

Plate 3 Redox cycling of αα-Hb causes endothelial cell cycle arrest and apoptosis. Bovine aortic endothelial cells were treated with (a, b) serum-free medium alone, or (c, d) medium containing 50 μM αα-Hb and H_2O_2-generating glucose oxidase. After 16 hours, cells were stained with Hoechst 33342, Alexa Fluor 488 annexin V conjugate, and propidium iodide (PI), and visualized by phase contrast and immunofluorescence microscopy (for detailed description see D'Agnillo, 2004). Hoechst 33342, a cell permeant blue fluorescent DNA dye, was used to visualize nuclear morphology. Alexa Fluor 488-annexin V conjugate was used to detect phosphatidylserine on the outer leaflet of the plasma membrane, an early apoptotic marker. PI, a red fluorescent nucleic acid dye, enters and stains cells with decreased plasma membrane integrity. Control cultures showed an intact monolayer with typical pale blue nuclei staining by Hoechst 33342 and no annexin V or PI staining (a, b). Treatment with αα-Hb and glucose oxidase caused cell rounding, detachment, and condensation of nuclei stained brightly by Hoechst coupled with annexin V plasma membrane staining (green), indicative of apoptosis (c, d). Glucose oxidase or αα-Hb alone did not produce these morphological changes (not shown). See Fig. 19.1.

Chapter 20

Renal Toxicity

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INTRODUCTION

Inevitably many recipients of hemoglobin-based oxygen carriers (HBOCs) will be elderly, with dehydration, chronic kidney disease, congestive heart failure, multiple myeloma or diabetes and microalbuminuria. These conditions put the kidney at risk for acute renal failure following hemorrhage, trauma or surgery (Mehta and Chertow, 2003), and these are the patients whose kidneys are most likely to be vulnerable to the potential toxic effects of HBOCs. Predicting whose kidneys are likely to be adversely affected by oxygen carriers requires more epidemiological studies of acute renal failure, like those that have recently appeared (Mehta and Chertow, 2003). Understanding the biological mechanisms involved in hemoglobin and HBOC toxicity for kidneys at risk for acute renal failure requires good animal models. Unfortunately, extrapolating from animal experiments to human acute renal failure has not always been successful; but animal experiments are yielding new insights and new ways to detect renal damage (Heyman *et al.,* 2002) (Han *et al.,* 2002).

HISTORY AND BACKGROUND

It has been recognized for many years that hemolysis or rhabdomyolysis causes kidney failure. Renal tubules became filled with hemoglobin or myoglobin pigment, hence the term 'pigment nephrosis'. Most research into the renal toxicity of hemoglobin has focused on the acute effects within hours of administering hemoglobin or heme to animals. In 1967, the observation that highly purified hemoglobin is less toxic than hemolyzed red blood cells (Rabiner *et al.,* 1967) led to a decade of optimistic reports that purified stroma-free hemoglobin did not damage kidneys in experimental animals. Careful storage after removal of phospholipids and endotoxinreduced toxicity (Feola *et al.,* 1988). Changes in urine flow, creatinine clearance and renal histopathology were used as indicators of deleterious outcomes. Relying on these measurements in animal experiments, investigators in the 1970s predicted that highly purified hemoglobin would not be toxic to humans.

Researchers were disillusioned by the first clinical trial of purified hemoglobin in healthy humans (Savitsky *et al.*, 1978). Human volunteers infused with purified hemoglobin developed hypertension, bradycardia, oliguria, and a transient fall in creatinine clearance. One of the eight paid volunteers had abdominal pain and costovertebral-angle tenderness (Savitsky *et al.,* 1978). How was it that human nephrotoxicity had not been predicted from the results of animal studies? Probably because the criteria for nephrotoxicity were less stringent in animals. Survival for 5 days

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after 70–76 per cent exchange of blood for stroma-free hemoglobin was an acceptable end-point in studies using rats (Devenuto *et al.,* 1977). In contrast, in well-hydrated humans, decreases in urine output or creatinine clearance lasting longer than 4 hours are deemed to be evidence of acute renal failure (Mehta and Chertow, 2003).

Two non-specific terms are used in describing hemoglobin nephrotoxicity. *Acute renal failure* is a clinical diagnosis based on a sustained decrease in glomerular filtration rate (GFR) that cannot be explained by hypotension or hypovolemia. *Acute tubular necrosis* is a histopathologic diagnosis associated with an acute decrease in renal function.

Three mechanisms were postulated to explain hemoglobin's renal toxicity: tubular obstruction with casts, tubular cell necrosis, and vascular constriction. Glomerular filtration of hemoglobin led to uptake of hemoglobin by proximal tubular cells. Hemoglobin caused cell necrosis. Casts formed from cell debris, and hemoglobin obstructed the tubules. Thus it was reasonable to presume that incorporating hemoglobin into a larger molecule would reduce toxicity by limiting its entry into glomerular filtrate. Subsequent studies have confirmed that macromolecules containing hemoglobin are much less likely to cause tubular necrosis, but renal vascular constriction remains a problem during administration of many HBOCs.

CURRENT STATE OF KNOWLEDGE

Renal hemoglobin transport and toxicity for tubular cells

Infused hemoglobin binds to haptoglobins, albumin and other proteins until total plasma hemoglobin exceeds approximately 1.5 g/l (Winslow, 1992). Protein-bound hemoglobin does not pass through glomerular capillary walls, but unbound free hemoglobin tetramers readily escape through the glomerular filter because, although they are similar in size to albumin, they are not charged at physiological pH 7.4 (albumin is excluded from filtration by its negative charge). Filtered hemoglobin binds to megalin/cubilin receptors in the luminal brush border of proximal tubule cells, and from there is transported into endosomes (Gburek *et al.*, 2002, 2003). In endosomes, hemoglobin is broken down to globin, heme and iron. Heme is lipophilic, which allows it to penetrate throughout the cell and the intracellular canalicular system. Heme is metabolized by heme oxygenase-1 and -2 (HO-1, HO-2) to carbon monoxide, iron and biliverdin. Ferritin binds the free iron, which is potentially toxic.

Heme produces cellular damage by stimulating redox reactions that lead to production of reactive oxygen species (ROS) and to oxidative denaturation of lipid, DNA, and cytoskeletal and other proteins (Gonzalez-Michaca *et al.*, 2004; see Chapter 18). Globin by itself is relatively innocuous (Winslow, 1992). Heme and iron, either directly or through ROS, increase the activity of hypoxia-induced-factor-1α, nuclear factor-κB (NF-κB), monocyte chemoattractant protein-1(MCP-1) and HO-1 (Kanakiriya *et al.*, 2003). NF-κB and MCP-1 exacerbate acute and chronic tubulo-interstitial inflammation (Chandel *et al.*, 2000; Nath *et al.*, 2001a), while HO-1 protects the kidney not only from the damaging effects of free hemoglobin but also from damage by hypoxia, hyperoxia, inflammation, toxic chemicals and oxidative stress.

Heme oxygenases

Heme oxygenases are rate-limiting for heme metabolism. Three HO variants have been described. HO-2 and HO-3 are constitutively expressed, but HO-3 has little heme-degrading capacity. HO-1, the inducible variant, is minimally expressed in the kidney, but its activity rises three-fold within 6 hours following a stroma-free hemoglobin (SFH) infusion (Lieberthal *et al.*, 2002). The site of maximum HO-1 expression varies with the nature of the stimulus (Rosenberger *et al.*, 2002). Paradoxically, inhibition of HO-1 prevents tubular damage to cultured cells or isolated tubules *in vitro* (Holt and Moore, 2001), but inhibition of HO-1 *in vivo* exacerbates injury to tubule cells and kidney function (Gonzalez-Michaca *et al.*, 2004). Doses of SFH that have no effect on HO-1+/+ mice produce severe acute renal failure in HO-1−/− knockout mice (Nath *et al.*, 2000b). HO-1 dampens expression of NK-κB and MCP-1 and increases expression of ferritin, which traps the potentially toxic iron released from hemoglobin (Gonzalez-Michaca *et al.*, 2004). High ferritin levels are associated with decreased risk for developing acute renal failure (Davis *et al.*, 1999). Cells overexpressing HO-1 have increased expression of p21, a cyclin-dependent kinase inhibitor that controls the entrance of quiescent differentiated cells into the cell cycle following tissue injury (Megyesi *et al.*, 2002). Carbon

monoxide from heme metabolism stimulates expression of p21. Upregulation of p21 prevents uncoordinated cell cycling and increases resistance to apoptosis (Gonzalez-Michaca *et al.*, 2004).

Hemoglobin-induced vasoconstriction could produce patchy hypoxia or ischemia in the kidney. Ischemia disrupts cells by depleting them of energy provided by oxidative phosphorylation. Reperfusion of ischemic tissue creates ROS. Much of our understanding of renal ischemia/reperfusion injury comes from experiments in which the renal artery is clamped for 30 to 60 minutes and then released to allow reperfusion. The ROS and hypoxia produced by ischemia/reperfusion trigger apoptosis and necrosis (Bonegio and Lieberthal, 2002), as well as proteolysis that releases heme from endogenous proteins such as cytochromes, nitric oxide synthase and other heme-containing enzymes. The toxic effects of heme from endogenous sources are similar to those produced by exogenous hemoglobin. HO-1 is upregulated after an episode of 'ischemia/reperfusion' (Shimizu *et al.*, 2000) just as it is following exposure to SFH.

Desferoxamine, an iron scavenger, reduces the structural and functional damage produced by SFH injections (Paller, 1988) and ischemia/reperfusion (Salahudeen, 2004). Desferoxamine probably protects by scavenging not only iron but also semiquinone radicals that are produced by heme-induced redox reactions. Desferoxamine also stimulates hydrolysis of halogenated quinones that are responsible for single-strand DNA breaks, reduces protein-derived radicals that are responsible for lipid peroxidation (Zhu *et al.*, 1998) and upregulates HO-1(Yang and Zou, 2001).

Histopathology

The histopathological effects of purified hemoglobin and HBOCs have only been reported for animal studies. Within 12 hours after a large dose of SFH, patchy damage to tubule epithelia appears (Somers *et al.*, 1997). In the proximal tubules, particularly S2 and S3 segments (Heyman *et al.*, 2002), endosomes are filled with dense matter, cells are denuded of their brush borders, and there is partial or total loss of epithelial organization with dilation of interspaces or necrosis. Casts obstruct tubule lumens, especially in the medulla. Ten days after receiving 0.96 g of stroma-free hemoglobin, tubular necrosis was most evident in juxtamedullary proximal tubules, and there was proliferation of smooth endoplasmic reticulum in other proximal tubule cells (Chan *et al.*, 2000). Apoptosis and vascular damage, which are important features in other forms of acute tubular necrosis (Bonegio and Lieberthal, 2002), were not described in these earlier animal studies. However, more recent observations indicate that heme causes G2/M arrest and apoptosis in cultured proximal tubular cells (D'Agnillo and Alayash, 2001; Gonzalez-Michaca *et al.*, 2004). Apoptosis will be an important indicator of hemoglobin toxicity in future studies.

Vascular engorgement and endothelial damage, especially in the outer medulla, are prominent features of ischemia/reperfusion and glycerol-induced acute tubular necrosis (Heyman *et al.*, 2002). These features have not been described in studies of hemoglobin toxicity, but might be found if looked for in the light of current knowledge about acute tubular necrosis.

HEMOGLOBIN TOXICITY TO RENAL VASCULATURE

Extravasation

Hemoglobin A and a number of HBOCs (diaspirin crosslinked, bovine polymerized, human polymerized, Hb-PEG5K10 and $\alpha\alpha$-Hb) rapidly enter endothelial cells *in vivo* (Baldwin, 1999; Baldwin *et al.*, 2003) and *in vitro* (Dull *et al.*, 2004). They disrupt endothelial cell function, in part at least, by scavenging intracellular nitric oxide (Faivre-Fiorina *et al.,* 1999; Toussaint-Hacquard *et al.,* 2003). SFH, $\alpha\alpha$-Hb and Dex-BTC hemoglobin increase the permeability of endothelial layers to macromolecules, decrease transepithelial electrical resistance, and create intercellular gaps and actin stress fibers. These changes develop over 3–4 hours after cultured endothelial cells are exposed to hemoglobin. Hemoglobin polymers (see Chapter 32, Hb-Polytaur and Hb-Polytaur$_n$) produce more damage than Hb-A (Dull *et al.*, 2004). Increased peritubular vascular permeability could lead to protein extravasation and altered tubular fluid reabsorption, which depends upon protein oncotic gradients across capillary walls. Unfortunately, there are no published reports of studies that examine hemoglobin effects on the permeability and structure of renal endothelial cells. However, we do know that crosslinked and smaller conjugated hemoglobins escape through peritubular capillaries into renal lymph within 30 minutes after beginning an exchange transfusion

(Matheson *et al.*, 2000, 2002). Following diafiltration to exclude molecules of less than 300 kDa, hemoglobin was not detected in lymph. Hemoglobin-induced vascular injury develops gradually, and therefore the full effect would not be seen for several hours after administration of the HBOCs. Most acute experiments using HBOCs or hemoglobin have been too brief to observe such changes.

Hemoglobin effects on vascular function

Our unpublished observations suggest that HBOCs increase renal vascular resistance, partly as an autoregulatory response to raised systemic arterial pressure and partly through direct action on renal arteries and arterioles. Nitric oxide scavenging probably initiates most of the acute vasoconstriction, but generation of vasoconstrictor ROS could also play a role. Vascular tone is determined by the balance between vasodilators such as nitric oxide, and vasoconstrictors such as angiotensin, the sympathetic nervous system, endothelin, eicosanoids and ROS. Removal of the vasodilator nitric oxide leaves vasoconstriction unopposed. Introducing an HBOC that reduces nitric oxide and increases ROS is bound to upset the balance among renal blood flow, GFR and tubular reabsorption that is essential for maintenance of fluid–electrolyte homeostasis (Schnackenberg, 2002).

HBOCs with low oxygen affinity might raise PO_2 in the cortex or medulla and alter the activity of oxygenases that are sensitive to changes in PO_2 above 40 mmHg (Baines and Ho, 2003). Increased HO-1 activity could counteract this effect by decreasing expression and activity of cyclooxygenase-2 and cytochrome p4504A enzymes that produce the vasoconstrictors PGE_2, PGI_2, thromboxane, 20-hydroxyeicosanoic acid and epoxyeicosatraenoic acid in the kidney (Botros *et al.*, 2002). Many factors interact to produce the vasoconstrictor response to HBOCs, and they may come into play at different times after HBOC infusion begins.

FUNCTIONAL CONSEQUENCES OF HEMOGLOBIN TOXICITY

Acute toxicity

The kidney is a highly vascular organ that depends upon adequate blood flow delivered to glomeruli at a high enough pressure to create glomerular filtrate. Maintenance of glomerular and tubular function depends totally on arterial pressure and renal vascular tone. Acute renal failure is in large part a vascular disorder, which accounts for the lack of correlation between tubular histopathology and overall renal function measured as GFR and urine production.

'Ultra pure' SFH administered slowly over 1 hour in amounts equal to or less than 2.5 g/kg did not raise serum creatinine in conscious rats (Somers *et al.*, 1997). Larger quantities of SFH raised serum creatinine two- to three-fold for several days (Somers *et al.*, 1997; Lieberthal *et al.*, 2002). Renal function is depressed transiently or for days, depending upon the quantity of hemoglobin infused. Loss of normal cell polarization reduces tubular solute and water reabsorption, and loss of adhesion molecules opens paracellular leaks in the tubule wall. Casts, back-leaks, decreased reabsorption and vasoconstriction all contribute to decreased urine flow and GFR.

Exchange transfusion with HBOCs that do not enter glomerular filtrate transiently increases GFR in experimental animals (Lieberthal *et al.*, 2002), probably as a result of changes in glomerular hemodynamics associated with decreased hematocrit and efferent arteriolar vasoconstriction. Acute tubular damage does not appear to be a problem.

Chronic renal hemoglobin toxicity

Although GFR returns to pre-treatment levels in the days immediately following a mildly nephrotoxic exchange transfusion, chronic and progressive damage may have occurred. GFR could return to normal due to hypertrophy of undamaged nephrons, while chronic inflammatory processes may have been set in motion that will ultimately lead to chronic renal failure (Basile *et al.*, 2001; Nath *et al.,* 2001). Gradual release of heme from HBOCs trapped in the kidney for days to weeks (Keipert *et al.*, 1994) could produce chronic tubulo-interstitial inflammation like that seen following repeated administration of hemoglobin to mice or repeated glycerol-induced rhabdomyolysis in rats (Nath *et al.*, 2000a). A single episode of ischemia/reperfusion in rats can produce chronic tubulo-interstitial scarring many weeks later, even though GFR and renal histology may appear normal 4–8 weeks after the initial injury (Basile *et al.*, 2001). The possibility that long-acting HBOCs might

induce chronic renal damage does not appear to have been examined. This issue is especially important, because it is likely that HBOCs may be administered more than once to critically ill patients (Creteur and Vincent, 2003).

ACUTE RENAL FAILURE

Classification of human acute renal failure

When it comes to assessing whether HBOCs decrease the likelihood of acute renal failure or cause acute renal failure, the important question is 'what is acute renal failure?' Simply defined, acute renal failure has occurred when there is an increase in serum creatinine of 8–125 μmol/l (0.3–0.5 mg/dl) above baseline within 48 hours of an intervention; however, no one has shown an association of transient changes in serum creatinine with morbidity or with likelihood of long-term recovery (Mehta and Chertow, 2003). Mehta and Chertow (2003) used epidemiological data to classify human acute renal failure under four domains: (S) susceptibility; (I) insult; (R) response; and (E) end-organ consequences. *Susceptibility* relates to the severity of pre-existing chronic kidney disease and risk factors. Risk factors include diabetes mellitus with microalbuminuria, dehydration, multiple myeloma, congestive heart failure, and decompensated cirrhosis. *Insult* is rated as known or unknown, and by proximity to the current assessment. *Response* is measured by change in urine output and estimates of GFR. *End-organ consequences* include failure of respiratory, cardiovascular, neurological, hematological and liver functions. These criteria could be used to assess the response to hemoglobin or an HBOC. For example, consider Savitsky's trial of hemoglobin in human volunteers with normal kidney function and no risk factors (Savitsky *et al.*, 1978). Susceptibility was grade 1. Insult was grade 1, because it was recent and known. Response was grade 2, because GFR decreased by more than 50 per cent and urine output decreased for 4 hours. There were no apparent end-organ consequences. According to the Mehta and Chertow classification system, the volunteers experienced clinical acute renal failure S1-I1-R2-E1 and recovered. Application of a classification such as this could help to clarify the effect of therapy on outcomes. It would also help in the design and implementation of clinical trials. Initial trials of an HBOC might be limited to patients with S1-I1-R1 renal failure. Later trials might include those with a higher risk of severe renal failure.

Acute renal failure may go through four phases of variable severity and duration. In the *initiation* phase, GFR drops rapidly. It may then recover or proceed to decrease more slowly in an *extension* phase, followed by a *maintenance* period of stable low GFR and then a gradual *recovery*. The duration of each phase depends on the nature and severity of the insult and the susceptibility of the individual (Lieberthal *et al.*, 2000). Evidence is accumulating that the effective therapies for each phase will be different. An HBOC might reduce risk if given in the pre-renal or initiation phase, but be detrimental if given in the extension, maintenance or recovery phases.

An HBOC given during the extension or maintenance phase might exacerbate acute renal failure by damaging endothelial cell continuity. Restoration of endothelial continuity is essential for recovery in animal models of acute tubular necrosis (Brodsky *et al.*, 2002; Yamamoto *et al.*, 2002).

Sepsis with endotoxic shock worsens the prognosis of acute renal failure (Mehta and Chertow, 2003). Heme-stimulated redox reactions enhance damage by bacterial endotoxin (D'Agnillo, 2004), therefore HBOCs may not be beneficial when sepsis complicates hemorrhagic shock. The effects of HBOCs on septic animals have been studied in acute experiments, all but one of which lasted 3 hours or less. Diaspirin-crosslinked hemoglobin (Creteur *et al.*, 2000; Sielenkamper *et al.*, 1997) or polymerized hemoglobin given to rats and dogs with septic shock, raised blood pressure and slightly reduced lactate levels which suggests improved oxygen delivery (Heneka *et al.*, 1997). Some of the effects on oxygen delivery may have been the result of hemodilution (Creteur *et al.*, 2001). Continuous low-dose infusion of pyridoxalated hemoglobin polyoxyethylene conjugate 20 mg/kg per hour for 20 hours into septic sheep raised their blood pressure with minimal effects on blood flow distribution other than increased pulmonary artery resistance (Bone *et al.*, 1998). Unfortunately, the effect of HBOC treatment on renal function during or after septic shock has not been reported.

Preconditioning

There is reason to expect that HBOC administration prior to surgery could reduce the risk of

acute renal failure. The benefit may come not only from hemodilution (Creteur *et al.,* 2001), but also from 'preconditioning' the kidney. A variety of treatments given before a period of prolonged ischemia reduce functional and structural damage. Preconditioning, which can be produced by brief periods of ischemia, ureteral obstruction or $SnCl_2$, is associated with induction of NOS and HO-1 (Bonventre and Weinberg, 2003) and ferritin (Davis *et al.*, 1999; Gonzalez-Michaca *et al.*, 2004). Hemoglobin increases expression of these protective factors, and should therefore act as a preconditioning agent if given prior to surgery.

Animal models of acute tubular necrosis (renal failure)

Animal models of acute tubular necrosis do not correlate well with human acute renal failure (Lieberthal *et al.,* 2000; Rosen and Heyman, 2001; Heyman *et al.,* 2002; Bonventre and Weinberg, 2003). Human acute renal failure may follow transient mild hypotension with blood pressure between 80 and 100 mmHg, but rat kidneys survive a blood pressure of less than 50 mmHg for 2–3 hours (Lieberthal *et al.*, 2000). Blood pressure must be less than 20 mmHg to induce renal failure in rats. In contrast, total renal ischemia for 30–60 minutes is often used to produce acute tubular necrosis in rats, but human kidneys tolerate up to 75 minutes of warm ischemia without acute renal failure after transplantation. Structural and functional changes similar to those observed in human acute renal failure have been produced by the combined effect of high-output heart failure with indomethacin and L-NAME to inhibit prostaglandin and nitric oxide production (Goldfarb *et al.*, 2001), or by combining endotoxin infusions with inhibition of nitric oxide synthase (Heyman *et al.*, 2000). HBOCs have not been tested in models such as these. Instead, several studies have used 30 minutes of ischemia with reperfusion and found that $\alpha\alpha$-crosslinked hemoglobin and raffinose-crosslinked hemoglobin given immediately after 30 minutes of ischemia did not exacerbate the effects on GFR (Paller, 1988; Lieberthal *et al.*, 2000).

Pig kidneys are anatomically more similar to human kidneys than are rat or dog kidneys. Experiments with swine may provide the best picture of human responses to HBOCs. Hess and MacDonald (Hess *et al.*, 1992) used SFH to resuscitate dehydrated swine after they were hemorrhaged to a blood pressure of 44 mmHg. Creatinine rose to 3 mg and returned to control levels after 5 days, which indicates a transient decrease in GFR. Lactated Ringer's solution resuscitated swine without an increase in creatinine. Other studies with swine (McNeil *et al.*, 2001; Knudson *et al.*, 2003) were of short duration, and did not examine renal function or pathology.

SUMMARY

Careful purification and storage of stroma-free hemoglobin reduces its nephrotoxicity; however, whenever the plasma hemoglobin concentration exceeds the binding capacity of plasma proteins, hemoglobin enters the glomerular filtrate and is reabsorbed into proximal tubule cells. Intracellular metabolic processes break hemoglobin down to heme and iron, which increase the production of ROS. Proximal tubular cells become disorganized, necrotic, and probably apoptotic. Cytokines NF-κB and MCP-1 are activated, and may contribute to tubulo-interstitial inflammation. Increased HO-1 diminishes the toxic responses.

Renal vascular resistance increases as hemoglobin scavenges the vasodilator nitric oxide, leaving the vasoconstrictor actions of angiotensin, endothelin, thromboxane and the sympathetic nervous system unopposed. Increased oxygen-dependent production of vasoconstrictor eicosanoids may contribute to vasoconstriction.

Incorporating hemoglobin in a molecule that is too big to be filtered prevents these nephrotoxic effects on proximal tubule cells, and may reduce but not entirely prevent vasoconstriction. There is little information about the effects of these large HBOCs on renal peritubular capillaries. We need this information because HBOCs have been shown to enter endothelial cells and alter the permeability of endothelial layers *in vitro* and *in vivo*. Alterations in renal vascular permeability can have serious effects on renal function. Damage to peritubular vasculature, especially in the medullary region, appears to be a critical factor in the progression of chronic renal damage following an acute episode. By disrupting endothelial continuity, an HBOC or its metabolites could exacerbate vascular damage and hinder recovery.

Few (if any) of the animal experiments with HBOCs replicate the conditions under which HBOCs are likely to be used with humans.

Experiments such as those with dehydrated and severely hemorrhaged swine done several decades ago with αα-Hb are an exception. The structural changes seen in human acute tubular necrosis are not reproduced by brief 30-minute episodes of renal ischemia. Therefore the effect of an HBOC on creatinine clearance measured for several hours after 30 minutes of renal ischemia does not reveal much about the response of a patient at risk for acute renal failure. HBOCs should be tested in animal models that simulate acute renal failure in humans, and the outcomes should be tested for days and weeks.

There is little information about the effects of HBOCs long term on renal structure and function. This information is needed because recent evidence shows that repeated exposure to hemoglobin induces chronic tubulo-interstitial changes in rat and mouse kidneys (Nath *et al.*, 2000b). Furthermore, in rats an episode of ischemic acute renal failure associated with a transient fall in GFR may precipitate progressive chronic renal failure appearing months later (Basile *et al.*, 2001, 2003; Nath *et al.*, 2001). Large hemoglobin polymers or conjugates remain in the plasma for longer than hemoglobin dimers and tetramers, and will probably expose the kidney to low levels of heme for days or weeks. These responses raise concerns about the long-term renal effects of HBOC administration.

FUTURE DIRECTIONS

Animal models of acute and chronic toxicity

Currently there is no information about the acute effects of HBOCs on the renal vasculature and their long-term effects on tubulo-interstitial inflammation and scarring. Nor is there information about the effects of HBOCs on animals with acute tubular necrosis that simulates the human disease. These deficits should be rectified by observing the acute and chronic effects of HBOCs in animal models that more closely mimic human acute renal failure. Micro cDNA gene arrays are now being used to follow the cellular responses to renal damage (Devarajan *et al.*, 2003). Gene arrays could help to identify markers for the toxic effects of hemoglobin and HBOCs. One such marker, KIM-1, provides a urinary marker of damage to proximal tubular cells that may help identify the early stages of acute renal failure (Han *et al.*, 2002).

Clinical trials

Uncomplicated acute renal failure following blood loss can now be treated with mortality rates of only 5–10 per cent without the use of HBOCs. This demonstrates how difficult it will be to demonstrate the added benefit of treatment with an HBOC in uncomplicated acute renal failure. When non-renal organ system failure accompanies acute renal failure, the mortality rate increases to 50–70 per cent (Mehta and Chertow, 2003). This mortality rate has not decreased in recent years.

Up until now, many specific interventions to prevent the progression of acute renal failure have not been successful and in some cases may have been detrimental. Included in the list of failed treatments are loop and osmotic diuretics, 'renal dose' dopamine, atrial natriuretic peptide, insulin-like growth factor, and endothelin receptor antagonists (Mehta and Chertow, 2003). These agents were tried because experiments with animal models of acute tubular necrosis and observations with normal human volunteers suggested that they should work. The unpredictable nature of human acute renal failure and the multiple clinical problems that often accompany it make the evaluation of new therapies with randomized control trials very difficult. It has taken decades to demonstrate that dopamine, which increases GFR and urine flow in normal humans and animal experiments, does not improve outcomes in human acute renal failure. Likewise, it may take years of carefully documented use of an HBOC to demonstrate that it is as effective in preventing acute renal failure as the gold standard – namely human erythrocytes. In human trials, HBOCs have been compared to albumin, gelatin, dextran or hydroxyethyl starch (HES), which were used as colloidal solutions to restore plasma volume. Both dextran and HES have been associated with instances of acute renal failure, although the evidence supporting these claims is not strong (Boldt and Priebe, 2003). Nonetheless, the possibility that they may be even mildly nephrotoxic can complicate interpretation of results comparing them with HBOCs. Current opinion advises that HES be used cautiously in patients at risk for developing renal dysfunction. Unfortunately, there are few data to indicate which patients are at risk or how much risk is posed by the use of HES.

The *sine qua non* for establishing evidence of HBOC efficacy must be risk assessment prior to treatment. This will be helped by using a

classification system such as that proposed by Mehta and Chertow (2003). Evaluation of protective factors such as HO-1 and ferritin levels may help to stratify risk for acute renal failure. Patients with preoperative serum ferritin levels less than or equal to 130 μg/l had a six-fold greater likelihood of developing acute renal failure compared to patients with levels above this value. Following the state of renal function by simple monitoring of serum creatinine and creatinine clearance will not suffice. New markers, such as urinary KIM-1, may help to detect the presence of acute renal failure prior, during and following administration of an HBOC.

Intensive research using analytical tools provided by molecular biology and genetics is providing stimulating new information about the mechanisms of experimental acute tubular necrosis in a variety of animals. Methods for simulating human acute tubular necrosis are being developed. These animal models and diagnostic tools will make for more effective pre-clinical testing of HBOCs. Epidemiological information about the risks for and outcomes of human acute renal failure that is beginning to appear will help in designing effective clinical HBOC trials.

EDITOR'S SUMMARY

Renal toxicity has been recognized as a danger of cell-free hemoglobin for decades. Severe renal failure occurs after soft tissue injury ('crush' injury) in which considerable myoglobin and hemoglobin are liberated into the blood stream and the normal mechanisms for removal of these proteins are overwhelmed. Some early studies of hemoglobin-based blood substitutes were plagued by renal failure, which was thought to be due to impurities in early preparations.

Modern research has shown that some hemoglobins (particularly those with small excluded volume) may penetrate renal tubular cells, where oxidative damage may occur, leading to renal failure. It is most likely now that larger molecules are less renal-toxic than small ones, but precise correlations between size and toxicity are not available.

Clinical trials that measure only creatinine clearance for a short time after infusion may not be sensitive enough to predict renal effects, particularly in patients who have risk factors for renal failure prior to dosing. More animal studies are needed, under more realistic conditions, to discover what products present the most risk and to further define the mechanisms of toxicity. More clinical studies in a broad range of patients are needed in order to discover what patients might be at risk, how to predict susceptibility to toxic effects, and how to treat them when they occur.

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Chapter 21

Hemoglobin and Neurotoxicity

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INTRODUCTION

On 28 October 2004, the Center for Biologics Evaluation and Research (CBER) Division of the Food and Drug Administration (FDA) published a draft *Guidance for Industry: Criteria for Safety and Efficacy Evaluation of Oxygen Therapeutics as Red Blood Cell Substitutes*. In it, the FDA listed eight 'largely unresolved safety-related problems' that have arisen 'during the preclinical and clinical development of the current generation of hemoglobin-based products', one of which is neurotoxicity. The question of neurotoxicity became particularly important when clinical trials in the 1990s suggested that free hemoglobin, entering the brain from the vasculature, could cause significant tissue damage, resulting in disability or death.

Our laboratory has had a long-standing interest in the possible neurotoxicity of hemoglobin. In the early 1980s we explored the possible role of hemoglobin in the etiology of familial idiopathic epilepsy (Panter *et al.*, 1984, 1985), hypothesizing that individuals who had deficient mechanisms for the clearance of free hemoglobin from intra- and extravascular sites would have increased tissue damage from hemorrhagic injury. Therefore, we examined the protein that binds and clears hemoglobin: plasma haptoglobin (Javid, 1978; Dobryszycka, 1997). These studies showed that individuals with familial idiopathic seizure disorders were more hypohaptoglobinemic than a control population with symptomatic seizure disorders (Panter *et al.*, 1984, 1985). Chronic hypohaptoglobinemia may have hindered their ability adequately to clear hemoglobin in the brain following injury that, under normal circumstances, would have been considered inconsequential. These data suggest that prolonged exposure to free hemoglobin *in vivo* may have profound effects on the structure and function of the central nervous system (CNS). This led us to examine additional biochemical, cell culture and *in vivo* studies of the possible neurotoxicity of hemoglobin. This chapter reviews recent literature that has reported the possible neurotoxic effects of hemoglobin-based oxygen carriers (HBOCs) or hemoglobin itself.

IN VITRO EXPERIMENTS

Iron is critical for the transport of oxygen to tissues, but the chemical properties that permit it easily to interact with and bind oxygen also allow it to participate in chemical reactions that oxidize and damage cellular components. As a result, the concentration of 'free' iron in plasma or cells is highly controlled by physiology, and in

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normal homeostasis iron is present only when bound to proteins or other molecules. When iron is decompartmentalized as the result of tissue injury, or when HBOCs are injected into the vasculature, the potential for oxidative injury is significantly increased. The CNS is particularly sensitive to this type of injury, in part because of its high concentrations of polyunsaturated fatty acids, which are susceptible to oxidation. In the 1980s, we determined that hemoglobin was a potent oxidant of CNS lipids in homogenates of brain and spinal cord (Sadrzadeh *et al.*, 1987), and that these effects were attenuated by iron chelators and antioxidants. In other biochemical experiments, we determined that haptoglobin, the hemoglobin-binding protein, could inhibit hemoglobin's pro-oxidant activity – a finding that was later confirmed by other investigators (Sadrzadeh *et al.*, 1984; Gutteridge, 1987).

In a subsequent series of experiments supported by the US Army, our laboratory utilized co-cultures of neurons and astrocytes to study hemoglobin-related neurotoxicity. Studies showed that hemoglobin was toxic to neurons at low micromolar concentrations, but astrocytes were unaffected (Regan and Panter, 1993). At very low concentrations – levels that would normally not cause neuronal injury – hemoglobin significantly exacerbated excitotoxic neuronal injury (Regan and Panter, 1996), which is a significant cause of neuronal death following stroke or traumatic brain injury. Thus at higher concentrations hemoglobin was neurotoxic itself, and at lower concentrations it increased neuronal sensitivity to excitotoxins. Additional studies determined that this neurotoxicity could be blocked by antioxidants such as Trolox C or 21-aminosteroids, or by iron chelators such as desferoxamine, or by prebinding hemoglobin to haptoglobin prior to addition to cultured cells (Panter *et al.*, 1994; Regan and Panter, 1993).

More recent experiments in cell culture have compared the neurotoxicity of human hemoglobin A_0 with that of human sickle cell hemoglobin (HbS). Despite HbS's increased liability and tendency to oxidize to methemoglobin, HbS-dependent neuronal damage was comparable to that of Hb A_0 except at higher concentrations of hemoglobin, at which HbS was more neurotoxic and generated more intracellular reactive oxygen species (ROS) (Vanderveldt and Regan, 2004). Other recent studies have also revealed that there is a fairly broad treatment window for blocking hemoglobin-dependent neurotoxicity in cell culture. In experiments using 2′,7′-dichlorofluorescin oxidation (an intracellular indicator of free radical production), a significant hemoglobin-dependent increase of ROS was not apparent until 4 hours of neuronal exposure to hemoglobin (Regan and Rogers, 2003). Treatment with either an antioxidant (U74500A) or iron chelators (desferoxamine and phenanthroline) significantly protected neurons, even when applied 8 hours after exposure to hemoglobin.

All these experiments were conducted in primary cultures of murine neurons plated on murine astrocytes (mixed cell cultures). However, another culture system, prepared with 'commercially available fetal rat mixed neural cells', was used to test for the neurotoxicty of another hemoglobin preparation, HBOC-201 (from Biopure®), which was compared to adult human hemoglobin (from Sigma®). Three different assays of potential neurotoxicity were used: an assay for cell proliferation, an assay for metabolic activity, and a cytotoxicity assay (Ortegon *et al.*, 2002). The concentrations of hemoglobin used were expressed in g/dl, and the concentrations utilized were 0.02, 0.2, 2.0 and 6.5 g/dl. HBOC-201 significantly inhibited proliferation only at the highest concentration. Cellular metabolism was inhibited and cytotoxicity was increased by human hemoglobin at 2.0 and 6.5 g/dl, but HBOC-201 had no effect at any concentration. These data cannot be compared with any of our previous published data in cell culture because of the significant differences between the two cell culture systems. In addition, the different cell culture systems use very different solutions of media. The difference between these two cell culture systems is especially obvious when comparing the effective concentration that kills 50 per cent of neurons (EC50) for 'human hemoglobin'. It was recently reported that the EC50 for human HbA_0 was 2.1 μM in mixed cultures of murine neurons and astrocytes (Vanderveldt and Regan, 2004); in cultured rat neurons, the EC50 appeared to be just under 6.5 g/dl. The difference is approximately 20 orders of magnitude. Just as different mouse strains or lines vary considerably in their neuronal vulnerability to oxidative injury, it appears that there is also a significant difference between rat and mouse neurons in their susceptibility to hemoglobin-dependent injury.

IN VIVO EXPERIMENTS

We have previously developed an animal model of subarachnoid hemorrhage in which lysed blood or purified hemoglobins were injected into the subarachnoid space of rat brain. The endpoints in this model were molecular – the induction of heat shock protein 70 (HSP70), which is thought to indicate cellular injury, or heat shock protein 32 (also known as heme oxygenase-1, HO-1), which has been interpreted as indicative of oxidative stress (Maines, 1992; Massa *et al*., 1996; Matz *et al*., 1996a, 1996b, 1996c; Turner *et al*., 1998). Purified hemoglobins did not induce HSP70 but did induce significant expression of HO-1; hence we interpreted these results to indicate that hemoglobin caused oxidative stress but not cellular injury (Massa *et al*., 1996). Lysed blood, on the other hand, induced focal expression of both HSP70 and HO-1 following subarachnoid injection, suggesting that cells in those focal areas were oxidatively stressed and injured (Maines, 1992; Massa *et al*., 1996; Matz *et al*., 1996b, 1996c). We believe it is likely that the hemoglobin in lysed red cells, perhaps in conjunction with other cellular elements, caused cerebral vasospasm that produced cerebral ischemia, which induced HSP-70. As in the cell culture experiments, the treatment of animals with an antioxidant (U-101033E) following hemoglobin injection reduced oxidative stress (HO-1 expression) and completely eliminated cell injury (HSP70 expression)(Turner *et al*., 1999).

The pathology of subarachnoid (SAH) and intracerebral hemorrhage (ICH) has been studied extensively in animal models, and the studies conducted by Dr R. Loch MacDonald using monkeys, dogs, and rats (SAH), and by Drs Julian T. Hoff and Guohua Xi in rats (ICH), are particularly relevant to this chapter. The two models have different endpoints of injury. In SAH the endpoint is cerebral vasospasm, and in ICH the endpoint is tissue edema and a behavioral deficit. Although both models rely on the injection of autologous blood to cause injury, it was demonstrated over a decade ago that cerebral vasospasm following SAH in primates was induced by oxyhemoglobin, but not by methemoglobin or bilirubin (MacDonald and Weir, 1991; MacDonald *et al*., 1991). More recently, it was shown that cerebral vasospasm in primates resolved when the blood clot that formed from a subarachnoid hemorrhage was removed, eliminating exposure to hemoglobin (Zhang *et al*., 2001). In these models, hemoglobin is a neurotoxic agent (Qureshi *et al*., 2001; Zhang *et al*., 2001; Sumann *et al*., 2002).

Both ICH and SAH models have been demonstrated to involve free radicals. In rats, ICH significantly increased protein carbonyl levels and the number of apoptotic neurons, and decreased both copper/zinc and manganese superoxide dismutase, 24 hours following the injection of blood (Wu *et al*., 2002). In dogs, SAH resulted in a 40–50 per cent decrease in the diameter of the basilar artery measured at 4, 7, 10, and 14 days post-injection of blood, and at 4 days following blood injection a significant increase in the concentration of malondialdehyde (a product of lipid peroxidation) was detected in both the basilar artery and the clot (MacDonald *et al*., 2004). In addition, salicylic acid was continually infused into the clot via a microdialysis probe, and the effluent fluid was examined for salicylate oxidation products – 2,3- and 2,5-dihydroxybenzoic acids (DHBA) – which are considered to be generated by free radical activity, specifically the hydroxyl radical. Both oxidation products were significantly increased on the fourth day following the injection of blood, and 2,5-DHBA remained elevated for 14 days (MacDonald *et al*., 2004). Thus, both ICH and SAH appear to involve the generation of reactive oxygen species.

TISSUE RESPONSE TO HEMOGLOBIN METABOLITES AND POSSIBLE NEUROPROTECTION

Both ICH and SAH have been demonstrated to affect the tissue levels of proteins involved in the metabolism of hemoglobin and its degradation products (heme and iron), and tissues were harvested at different timepoints following ICH or SAH and examined for these molecules. After ICH, heme oxygenase-1 (HO-1), the inducible form of HO in brain, was significantly elevated at 1, 3 and 7 days following injection of blood, but by 28 days its tissue concentration had returned to normal (Wu *et al*., 2003). One of the products of HO-1 activity is iron, and non-heme iron was significantly elevated as discerned by both iron histochemistry and non-heme iron quantitation at 3, 7, 14 and 28 days following blood injection. Three days following ICH, the tissue concentrations of both transferrin and the transferrin receptor were significantly elevated, and the tissue

concentrations of both the light and heavy ferritin subunits were significantly increased even at 28 days post-ICH (Wu *et al.*, 2003). Following SAH in monkeys, the tissue concentration of both the heavy ferritin subunit and HO-1 were significantly elevated at 3, 7 and 14 days (Ono *et al.*, 2000). Therefore, the tissue levels of proteins involved in the breakdown of hemoglobin and its metabolites are upregulated by both ICH and SAH, but whether these increases in tissue concentrations confer neuroprotection is uncertain.

Investigators have used two different approaches to determine whether a specific protein might protect against hemoglobin-dependent neurotoxicity. The first method is to increase the levels of the specific enzyme or protein, and the second method is to alter the genome to modify the expression of that specific enzyme or protein. In one study, confluent cultures of glia were exposed to hemoglobin for 2 hours (an exposure that does not result in toxicity), and afterwards it was determined that HO-1 was significantly induced (Regan *et al.*, 2000). These cells were then exposed to hemin for 23–30 hours, and cell death was significantly inhibited. Therefore, induction of HO-1 was protective, and additional experiments showed that if HO-1 was inhibited by tin-protoporphyrin IX, or if protein synthesis was blocked by cycloheximide, neuroprotection was lost (Regan *et al.*, 2000). In the same cell culture system, it was determined that a short exposure to non-toxic levels of hemoglobin, heme, or ferrous sulfate-induced ferritin was also found to block heme-dependent cellular toxicity by 85 per cent (Regan *et al.*, 2002).

Two studies have used viral vectors to transfect cells and induce HO-1 levels in tissue. The first, performed in the astrocyte cultures mentioned above, demonstrated that an adenoviral vector increased the expression of HO-1 and protected the cells from heme-dependent toxicity (Teng *et al.*, 2004). The second study was performed *in vivo* in rats and also utilized an HO-1 expressing adenovirus, which was injected into the cisterna magna (Ono *et al.*, 2002). One day after the viral injection the basilar artery was removed, and HO-1 messenger RNA and protein were found to be significantly increased in the arterial adventitia. In other rats that had been injected with the viral vector, when increasing concentrations of hemoglobin were applied to the basilar artery *in vivo*, contraction (vasospasm) was significantly decreased (Ono *et al.*, 2002). These two studies confirm that HO-1 protects against hemoglobin- or heme-dependent toxicities in central nervous system-relevant models.

The second approach – to alter the genome of an animal to increase or decrease the expression of a protein – was accomplished using mice in which the gene for hemeoxygenase-2 (HO-2) was specifically knocked out (HO-2−/−). When cortical neurons from HO-2−/− mice were cultured and exposed to hemoglobin, cell death was significantly attenuated and oxidative stress, evaluated using a fluorescent indicator of the generation of ROS, was decreased (Rogers *et al.*, 2003). In a more recent report, it was demonstrated that neurons from HO-2−/− mice were less vulnerable to hemin-mediated toxicity, and the generation of ROS and protein carbonyls, an indicator of intracellular ROS-mediated oxidation, were both significantly decreased (Regan *et al.*, 2004). Thus HO-2, which is non-inducible and constitutively produced in neurons, may actually contribute to hemoglobin- or heme-mediated neuronal toxicity, perhaps by generating intra-neuronal iron, one of the products of its activity.

Iron plays a role in neural injury as a potent pro-oxidant, whether it be in ICH, SAH, traumatic brain injury or ischemia. Acutely, iron can participate in chemistry that results in lipid, protein or nucleic acid oxidation, all of which may contribute to aging and neurodegenerative disorders (Zecca *et al.*, 2004). This is confirmed by studies using antioxidants, which can scavenge ROS generated by iron, and others using the high-affinity iron chelator, desferoxamine (DFO), which binds iron and prevent its participation in chemical reactions that generate ROS. For example, potent antioxidants of the 21-aminosteroid class (U74500A) protected against hemoglobin-dependent neurotoxicity in cultured neurons, and the neuroprotection was effective even when the compound was added 8 hours following the beginning of hemoglobin exposure (Regan and Rogers, 2003). A second compound of this class, U74006F, was demonstrated to minimize vasospasm in a preclinical model of SAH in rabbits (Vollmer *et al.*, 1989). In fact U74006F, or Tirilizad, was tested in Phase III clinical trials for aneurysmal subarachnoid hemorrhage (Baldwin *et al.*, 2004); however, product development was discontinued for this indication.

Desferoxamine, which chelates iron with an affinity of 10^{31}, has been demonstrated to diminish

vasospasm following SAH in rabbits (50 mg/kg, subcutaneously every 8 hours) (Vollmer *et al.*, 1991). In addition, in a rat model of ICH, DFO (100 mg/kg intraperitoneally over 12 hours) significantly attenuated cerebral edema and the accompanying neurological deficits (Nakamura *et al.*, 2004). Another iron chelator, deferiprone, was demonstrated to attenuate vasopasm following SAH in rabbits (Arthur *et al.*, 1997). Just recently, we have completed a study demonstrating that tissue damage from focal ischemia/reperfusion injury in rats could be reduced 60 per cent by pretreatment with intranasal desferoxamine (three doses of 6 mg each). Therefore, it may eventually be possible to treat hemoglobin- or heme- or iron-dependent neural injury or neurodegeneration by intranasal administration of desferoxamine or other compounds.

CLINICAL EXPERIENCE WITH HBOCs

Perhaps the best evidence that hemoglobin is neurotoxic is provided by the neurosurgery literature discussing either traumatic brain injury (TBI) or spontaneous subarachnoid or intracerebral hemorrhage. TBI is frequently accompanied by subarachnoid hemorrhage, and the early treatment of TBI focuses on three areas: stabilization of respiratory function, stabilization of circulatory function, and hemorrhage control. Effective treatment includes drilling burr holes in the skull to remove hematomas, which are associated with increased tissue edema, increased intracranial pressure, and decreased cerebral blood flow and tissue oxygenation (Sumann *et al.*, 2002; Mattioli *et al.*, 2003; Rosenfeld, 2004).

Vascular components can also come into direct contact with brain tissue following bleeding caused by spontaneous intracerebral or subarachnoid hemorrhage, usually produced by the rupture of an aneurysm (Qureshi *et al.*, 2001; Ogungbo *et al.*, 2003). Clinically, subarachnoid hemorrhages are accompanied by significant vasospasm, which occurs in both early (3–4 days following hemorrhage) and late (7–14 days following hemorrhage) stages. Early vasospasm, in particular, is a highly significant predictor of cerebral infarction, neurological worsening and poor outcome in patients with aneurysmal subarachnoid hemorrhage (Baldwin *et al.*, 2004).

Arteriovenous malformations (AVM) are another cause of intracerebral hemorrhage, and patients frequently present with 'seizures, headache, or neurological deficits from an acute intracranial hemorrhage – in the vast majority of cases the most dreaded event' (Soderman *et al.*, 2003). AVMs are particularly difficult to treat because of the significant rate of rebleeding. Regardless of origin, hemorrhage is associated with increased morbidity and mortality, and two recent review articles on spontaneous intracerebral hemorrhage both recommend early evacuation of the clot to 'reduce the mass effect, block the release of neuropathic products from the hematoma, and prevent prolonged interaction between the hematoma and normal tissue, which can initiate pathologic processes' (Qureshi *et al.*, 2001). It is very likely that hemoglobin, in part, plays a significant role in those 'pathologic processes'.

There are currently two HBOCs in Phase III clinical development: Polyheme®, being developed by Northfield Laboratories, and Hemopure®, being developed by Biopure. A third hemoglobin-based product, Hemospan®, is being developed by Sangart, Inc. (Winslow, 1992; Moore, 2003); this has progressed through Phase I clinical trials and is approved to move to Phase II. Collectively, the compounds above and a Baxter product that is no longer in clinical development, DCLHb, have been infused into several thousand patients with encouraging results. They seem to be safe, and their use conserves blood products and reduces patient exposure to allogeneic blood. However, with one exception, these studies have been performed in very controlled situations – i.e., perisurgical hemodilution. The exception is Northfield's recently completed trauma trial, in which Polyheme® was used as a resuscitation fluid in urgent blood loss (penetrating trauma without head injury) (Gould *et al.*, 2002). The patients in the above trials had intact blood–brain barriers that would serve to block the movement of free hemoglobin from the vasculature to the brain, preventing hemoglobin-, heme- or iron-dependent neurotoxicity. However, the real value of an HBOC will be in combat casualty care or emergency medicine, which are uncontrolled situations, many of which involve TBI. In these cases, the blood–brain barrier is open and free hemoglobin will enter the brain.

The possibility that free hemoglobin inside the human brain, outside the vasculature, is deleterious was especially highlighted by the results of clinical trials. In 1999, two articles were published that had a major impact on efforts to develop suitable HBOCs. The first described a controlled safety study of DCLHb in stroke patients (Saxena

et al., 1999). DCLHb, a product being developed by Baxter Healthcare, had demonstrated some ability to improve outcome in animal models of stroke (primarily in rats); therefore, Baxter moved toward a stroke trial in humans (Winslow, 2000). Within 18 hours of stroke, patients were infused with 12 doses of hemoglobin at 6-hourly intervals over the next 72 hours at dosages of 25, 50 or 100 mg/kg. Unexpectedly, and tragically, the rate of adverse outcome in patients who received DCLHb was double that of the saline controls, using three different scales of outcome assessment. More important, the rate of death in patients who received DCLHb was triple that of the saline controls.

The second article described the results of a Baxter-sponsored Phase III multicenter clinical trial in which DCLHb was used to treat patients with severe traumatic hemorrhagic shock (Sloan *et al.*, 1999). The historical mortality in this patient group was 40 per cent, and the aim of the trial was to reduce mortality to 30 per cent. Outcome was evaluated 28 days following treatment. Mortality in the DCLHb-treatment arm increased to 46 per cent, and that in the control arm (saline) decreased to 17 per cent. Although there are a number of possible reasons for the increased mortality in the treatment arm, one may be that patients with head injury who were treated with hemoglobin instead of saline were more likely to die; these patients accounted for 29 per cent of the deaths in the DCLHb-treatment arm, compared to 12 per cent of the deaths in the control arm (Sloan *et al.*, 1999, 2001, 2002).

The blood–brain barrier is disrupted following both stroke and TBI, and if an HBOC is present in the vasculature, it will enter the brain. Results from the two trials described above suggest that the presence of free hemoglobin in human brain may be toxic. This possibility is supported by studies conducted by us and reported in a series of papers demonstrating that hemoglobin was toxic to neurons in culture. These studies, first funded by the US Army and later by NHLBI, suggested that hemoglobin might be neurotoxic. This is exactly our interpretation of the two clinical trials described above; the compromised blood–brain barrier in stroke patients and trauma victims permitted free DCLHb to enter the brain from the vasculature, and as a result the outcome for these patients was significantly compromised. Now, 10 years later, two other companies developing HBOCs (Biopure and Northfield) have proposed conducting trauma trials, one in which the HBOC will be administered in the hospital setting (in the emergency room – Biopure), and one in which the hemoglobin will be administered in a pre-hospital setting (at the accident scene or in the ambulance – Northfield). Yet there is no evidence that these two different HBOCs are less neurotoxic than DCLHb.

The trials conducted by Baxter were terminated because increased mortality was observed in the treatment arm. The preclinical and clinical data strongly suggest that this mortality occurred, in part, because DCLHb came into direct contact with the brain. Northfield is working with the US Army to submit a treatment investigational new drug (treatment IND) application to the FDA (Moore, 2003) that will permit the administration of Polyheme® to battlefield combat casualties suffering hemorrhagic shock. The likelihood is high that in many of these casualties the blood–brain barrier will be breached. Unfortunately, the literature indicates that resuscitation with an HBOC under these circumstances may have detrimental, or even lethal, effects.

EDITOR'S SUMMARY

One of the chief applications for blood substitutes is in trauma, especially on the battlefield and in instances where blood is not immediately available. In these cases, it is not usually apparent what the extent of injury is, and often patients are comatose. Thus it is not often clear when the blood–brain barrier has been breached, allowing the blood substitute to enter extravascular spaces.

Research in a variety of cell culture and *in vivo* models has shown that cell-free hemoglobin is neurotoxic. The mechanisms of this toxicity appear to include oxidative reactions involving free iron, and the penetration of heme into vascular or parenchymal cells. This is supported by both circumstantial and direct measurements. What is not as clear is to what extent chemical or genetic modification

of cell-free hemoglobin, or its encapsulation in liposomes, can overcome this toxicity. Furthermore, some experimental systems demonstrate that cell-free hemoglobin can induce protective enzymes, including heme oxygenase and other heat-shock proteins. Thus the clinical effects of any new product are not easily predicted based on current knowledge, and each must be tested in a set of models to assure that it is not as toxic as unmodified hemoglobin.

That the concern over neurotoxicity is not limited to *in vitro* and cell culture systems is suggested by several recent disappointing clinical trials where morbidity and mortality were increased in the treatment arms of Phase III clinical trials, even though the products studied had, in some cases, been shown to be neuroprotective in some animal models. Clearly this is an area of great importance to the further development of both hemoglobin- and perfluorocarbon-based products.

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Chapter 22

The Role of Inflammation in the Toxicity of Hemoglobin-Based Oxygen Carriers

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INTRODUCTION

Transfusion of hemoglobin (Hb)-based blood substitutes, designed for their plasma expansion and oxygen transport capabilities, has resulted in some major problems, such as organ dysfunction, during clinical trials. Experimental evidence demonstrates that these hemoglobins damage tissue by producing highly reactive oxygen species (ROS). Although cell-free Hb may present a low risk to people with normal redox status, patients who are sick and have a poor antioxidant status may be at risk. Oxidative damage is particularly dangerous in the microcirculation because excess leakage of plasma components into the interstitium will disturb the fluid balance between blood and tissue and alter the kinetics of delivery of intravascularly injected drugs, and endogenous enzymes and hormones, to various tissues. In this review, the effects of Hb-based blood substitutes (HBOCs) on the body's inflammatory response will be described; in particular, their effects on mast cells, eosinophils and complement. These responses will be considered in terms of their impact on surrounding vascular and non-vascular tissues. Most of the responses reported involve the intestinal mucosa, which is not surprising considering the plethora of immune cells that reside in that area. Several possible mechanisms will be postulated to explain how HBOCs produce the observed oxidative damage, and various methods to reduce the deleterious effects of blood substitutes *in vivo*, using this information, will be evaluated.

THE INFLAMMATORY RESPONSE

Cellular responses

Inflammation is the body's response to foreign invaders, to rid the body of the invaders and to dispose of damaged tissue so that healing can occur. Sometimes the response does more harm than good, and results in repeated injury and persistent inflammation. It is the white blood cells (leukocytes) that are the functional cells for inflammation and immunity. On maturation most leukocytes are released into the blood and other extracellular fluids, such as lymph, after which they circulate to different organs and are able to penetrate the tissue. The leukocytes involved in inflammatory responses, basophils, eosinophils, neutrophils and macrophages, together with a tissue cell, called a mast cell, act on blood vessels

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and smooth muscle to initiate inflammatory responses in the tissue.

Cytokines

Activated neutrophils release prostaglandins which cause microvascular leakage, possibly by stimulating mast cells to release histamine; prostaglandins also attract leukocytes. Neutrophils and macrophages release proteases when stimulated, which cause microvascular leakage, attract more neutrophils and eosinophils, and degrade basement membranes, which support endothelial and epithelial cell layers. Activated mast cells can release interleukin-5 (IL-5), which attracts eosinophils – particularly to Peyer's patches in the intestinal mucosa (Mishra *et al.*, 2000). Other products produced by mast cell degranulation may then stimulate the eosinophils to produce leukotrienes as well as high concentrations of reactive oxygen species (ROS) (Raible *et al.*, 1992; Zeck-Kapp *et al.*, 1994; Sartor, 1995). In fact all cells produce ROS, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxide (OH^-) to some degree, as a result of respiration. These oxidants are normally removed by oxidant scavengers, such as superoxide dismutase, catalase, and vitamins E and C. Under some circumstances, production of oxidants exceeds the rate at which they can be removed by scavengers. This usually occurs in cells with a large number of mitochondria (the organelles that participate in cell respiration), such as the cells that line the intestine and the airways (epithelial cells).

Complement

The inflammatory response can be triggered by exogenous or endogenous agents. In addition, sometimes an exogenous agent can activate endogenous inflammatory mediators, such as complement C3, which is found in the plasma. This mechanism of activation is called the 'alternate pathway' of complement activation, as opposed to the classical complement pathway. Complement C3 gives off an anaphylatoxin fragment, C3a, which causes release of histamine from mast cells in the tissue, and the histamine increases microvascular leakiness. Complement C3 also activates complement components C6–C9, leading to generation of bradykinin and fibrinopeptides, that increase microvascular permeability and attract leukocytes to the site of injury.

BLOOD VESSELS

Inflammatory mediators, such as histamine and serotonin (from mast cells), bradykinin (generated by activation of the complement cascade), prostaglandins (from neutrophils) and oxidants (from activated neutrophils mast cells and macrophages), act directly on the small blood vessels (venules) to increase their permeability. These chemicals act on the endothelial cells that line the blood vessels so that the junctions between the cells become wider at focal points – i.e., gaps form. It is generally thought that the inflammatory mediators bind to receptors on the endothelial cell membrane which then signal to the cell, via various second messengers, to contract the actin fibers forming the cell's cytoskeleton; thus the cells actively contract away from each other to form the gaps (Majno and Palade, 1961). However, more recent research (Baldwin and Thurston, 1995) suggests that another mechanism may be involved in gap formation; that is, some inflammatory mediators may disrupt the actin fibers that are arranged circumferentially at the edges of the cells, and at the same time may disrupt the junctional adherence proteins (cadherens) that help to hold the cells together. The cadherens are linked to the actin fibers by other molecules called catenins. Thus gaps form between the cells at the sites of disruption. These gaps are of such a size that not only water but also plasma proteins, such as albumin and gamma-globulin, can leak out of the blood vessels into the tissue. This leakage accounts for the edema (or swelling) that is seen in inflammation.

Reactive oxygen species

There is some experimental evidence to support the idea that modified hemoglobins, injected *in vivo*, can cause tissue damage and organ dysfunction by producing reactive oxygen species (ROS) (Motterlini *et al.*, 1995; Hess, 1999; Baldwin and Wiley, 2002). Reactive oxygen species, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are formed because hemoglobin is susceptible to oxidation and autoxidation. Some hemoglobin-based blood substitutes have been shown to oxidize more readily than hemoglobin contained in red blood cells in response to chemical modifications aimed at lowering oxygen affinity (Yang and Olsen, 1989). Although cell-free hemoglobin may present a low risk to people with normal

redox status, patients who are sick and have a poor antioxidant supply may be at higher risk. Oxidative damage is particularly dangerous in the microcirculation because gaps form between the endothelial cells resulting in excess leakage of plasma components into the interstitium. Such leakage disturbs the fluid balance between blood and tissue and alters the kinetics of delivery of intravascularly injected drugs and endogenous enzymes and hormones to various tissues. Thus it is important to remember that blood substitutes should not just be considered as oxygen carriers; it is essential that their antioxidant capacity is adequate, because reperfusion of the circulation after loss of blood triggers production of ROS.

The intestinal mucosa

Hemorrhagic shock is the most obvious condition that would benefit from a readily available blood substitute. One vital organ that is particularly adversely affected during hemorrhagic shock is the intestine. Shock results in loss of gut mucosal integrity, allowing translocation of bacteria and endotoxins into the circulation, producing a systemic inflammatory response. The intestine is also important with respect to the immune system because the mucosa contains Peyer's patches, or organized aggregates of lymphoid tissue (mainly B-cells and T-cells) in between the villi. Peyer's patches play a key role in the initiation and expression of mucosal immunity. In addition, there are many resident inflammatory cells, such as neutrophils, eosinophils and mast cells, within the intestinal mucosal villi. Thus, in the intestinal mucosa any blood substitute that has seeped through the damaged walls of the blood vessels will have direct access to the body's immune system.

BLOOD SUBSTITUTES AND INFLAMMATION

Mast cells

Mast cells mature in all vascularized, peripheral tissues. They are especially numerous in the skin, and in the gastrointestinal and respiratory tracts. Within connective tissues such as the mesentery the mast cells are predominantly located adjacent to small blood vessels, especially postcapillary venules. Mast cells in gastrointestinal or respiratory tract mucosa occur both in the vicinity of small blood vessels and at sites distant from these vessels. Under electron microscopy mast cells can be identified by their granules, made electron dense by staining the sections with uranyl acetate and lead citrate (Figure 22.1a). Vacuoles are seen in degranulating mast cells (DMC, arrows), and some areas of cytoplasm appear to be disintegrating, perhaps because a granule has just been released. This electron micrograph shows a section through the intestinal mucosa of a rat 8 minutes after a bolus injection of diaspirin crosslinked hemoglobin (DBBF-Hb, Walter Reed Army Institute of Research, Washington DC). Note the widened junctions between the epithelial cells (E) and their long cytoplasmic projections. A capillary (C) is also visible.

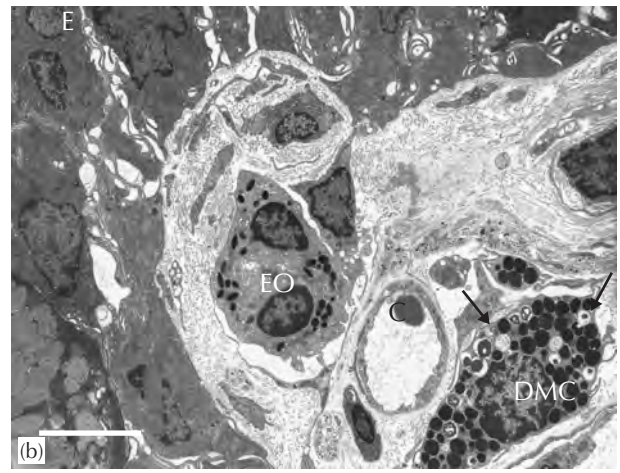

Figure 22.1 (a) Electron micrograph of an ultrathin section through the intestinal mucosa of a rat 8 minutes after a bolus injection of αα-Hb. Mast cells can be identified by their granules, made electron dense by staining the sections with uranyl acetate and lead citrate. Vacuoles are seen in degranulating mast cells (DMC, arrows). Note the widened junctions between the epithelial cells (E), and their long cytoplasmic projections. A capillary (C) and eosinophil (EO) are also visible. Scale bar = 5 microns.

Mast cells degranulate to produce a wide range of bioactive mediators, many of which are preformed (such as histamine, heparin and a spectrum of neutral proteases) and stored in an active state, thus allowing an immediate effect upon release. Certain proteases that are released can attack type IV collagen, which is found in basement membranes (Patrick *et al.*, 1988). Other mediators, such as leukotrienes, prostaglandin

and cytokines (TNF-α, IL-4, IL-5 and IL-6), are synthesized on mast cell stimulation and released on degranulation. Several studies have shown that both connective tissue and mucosal mast cells are stimulated to degranulate when any one of a range of HBOCs is injected into the circulation of rodents (Baldwin *et al.*, 1998, 2002a; Baldwin, 1999). Recent experiments, using male Sprague–Dawley rats, showed that the number of degranulated mast cells per villus in the intestinal mucosa (light microscopic sections) was significantly increased after bolus injection of diaspirin crosslinked hemoglobin (DBBF-Hb) or polyethylene glycol (PEG)-conjugated Hb (PEG-Hb, Enzon Inc., Piscataway, NJ) compared to buffered saline (Baldwin and Valeski, 2003). The cause of this degranulation is probably generation of ROS by the HBOCs. In fact excess ROS have been demonstrated in the intestinal mucosa of rats, using dihydrorhodamine 123, a fluorescent probe, following bolus injection of diaspirin crosslinked hemoglobin (DBBF-Hb) (Baldwin *et al.*, 2002b, 2002c).

Three possible mechanisms can account for the propensity of modified Hbs to form ROS (see Chapter 18). First, the ferrous and ferric forms of Hb can react with hydrogen peroxide (H_2O_2), produced by neutrophils or macrophages, to form the highly reactive ferryl intermediate $HbFe^{4+}$,and this may lead to heme degradation and cytotoxicity. A second possible redox mechanism involves the release of free iron from hemoglobin after oxidative damage. The free iron then catalyses the reaction between H_2O_2 and superoxide (O_2^-), both produced by phagocytes, to form the hydroxyl radical (OH^-) by the Fenton reaction. Thirdly, nitric oxide can react with oxygen free radicals, or with hemoglobin, to produce more ROS. The Fenton reaction appears to play a role in hemoglobin-induced mast cell degranulation because when rats were pre-treated with an iron chelator, phosphorothioate oligodeoxynucleotide, composed of 10 consecutive cytidines (PS-ODN), prior to bolus injection of DBBF-Hb, the mast cell degranulation was inhibited.

Eosinophils

Eosinophils are a type of white blood cell (comprising about 1–4 per cent of the peripheral leukocytes) that is recruited from the peripheral circulation into the tissue, and is especially prominent at sites of allergic reactions and parasite infections. These immune cells contain a bi-lobed nucleus and ellipsoid cytoplasmic granules. The granules each have an electron-dense, crystalline nucleus, and are composed of toxic, cationic proteins. When activated, eosinophils disrupt and deposit their toxic proteins into the surrounding tissue. Mast cells produce a chemotactic factor for eosinophils. The electron micrograph of rat intestinal mucosa (Figure 22.1a) shows an eosinophil (EO) located close to a degranulating mast cell. However, eosinophils commonly reside in the intestinal mucosa even in the absence of inflammation. Figure 22.1b shows such an eosinophil (→) beneath the intact epithelium (E) of an animal that received a bolus injection of buffered saline. Eosinophils can also be easily recognized by light microscopy when the tissue is stained with diaminobenzoate (DAB) and toluidine blue because their peroxidase granules stain brown, in contrast to the mast cell granules, which stain blue. A light micrograph of part of an intestinal villus cross-section containing eosinophils is shown in Figure 22.2 (arrows). This animal had received a bolus injection of αα-Hb 15 minutes prior to fixation. The epithelial cells (E) are detaching from the basement membrane and the inter-epithelial junctions are considerably widened at their basal aspect.

The mean cell counts (eosinophils and degranulated mast cells) for these animals are shown in Table 22.1. For both eosinophils and degranulated mast cells, the cell counts differed significantly between saline and αα-Hb, αα-Hb and

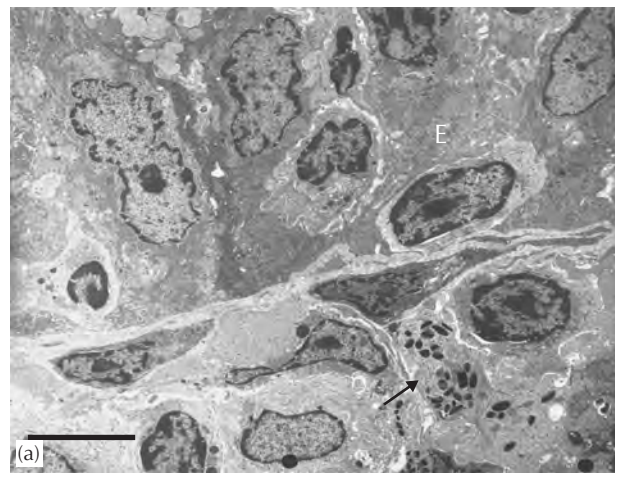

Figure 22.1 (b) Electron micrograph of an ultrathin section through the intestinal mucosa of a rat 8 minutes after a bolus injection of buffered saline, showing an eosinophil (→) beneath the intact epithelium (E). Scale bar = 5 microns.

Hb-PEGP5K10 (Enzon), and saline and Hb-PEGP5K10 ($P < 0.01$). These results support the hypothesis that bolus injection of αα-Hb and Hb-PEGP5K10 causes mucosal mast cell degranulation, resulting in recruitment and activation of eosinophils. Activated mast cells can release interleukin-5 (IL-5), which attracts eosinophils, particularly to Peyer's patches (Mishra *et al.*, 2000). Other products produced by mast cell degranulation may then stimulate the eosinophils to prodce leukotrienes and ROS (Raible *et al.*, 1992; Zeck-Kapp *et al.*, 1994; Sartor, 1995). The ROS and other products released by eosinophils may be partly responsible for the epithelial disruption observed near the Peyer's patches of rats injected with some HBOCs. An alternative hypothesis is that since eosinophils are antigen-presenting cells, they may propagate immune responses by presenting antigen (Hb products) to T-helper 2 cells. The T-helper 2 cells could then release IL-5, which would aid in the recruitment of more eosinophils.

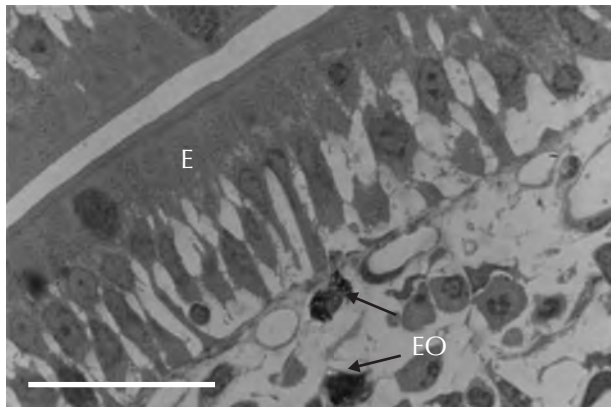

Figure 22.2 Light micrograph of a thick section through the intestinal mucosa of a rat 15 minutes after a bolus injection of αα-Hb. Eosinophils can also be easily recognized by brown diaminobenzoate staining of their peroxidase granules. The epithelial cells (E) are detaching from the basement membrane and the inter-epithelial junctions are considerably widened at their basal aspect. Scale bar = 25 microns.

Complement

Components of the complement system are acute phase reactants, increasing in concentration quickly after initiation of an inflammatory response, and participating in the response. It is known that the complement cascade is activated in trauma patients, with or without hemorrhagic shock (Gallinaro *et al.*, 1992; Moore, 1994). To determine whether complement plays a role in hemoglobin-induced injury of the rat intestinal mucosa, experiments were performed on anesthetized rats. In this study the rats were given a bolus injection of αα-Hb (10 mg/ml) and 1 hour later the intestinal mucosa was tested for *in vivo* deposition of complement component C3 (Valeski *et al.*, 2004). Frozen tissue sections of intestinal mucosa were probed with an antibody against complement C3, followed by a fluorescent secondary antibody, and examined by epifluorescence microscopy. Examination by incidence fluorescence microscopy did not identify a significant accumulation of complement C3 in the intestinal mucosa of the rats. The results suggest that complement does not play a significant role in αα-Hb-induced injury of the rat intestinal mucosa.

In another study, in which complement activation by liposome-encapsulated Hb (LEH) was investigated, a positive result was obtained (Szebeni *et al.*, 1997). The LEH products were prepared as described by Farmer and Gaber (1987). Various LEH and corresponding empty liposomes were incubated with normal human sera, and several markers of complement activation, such as serum levels of complement components and membrane deposition of C3b, were measured.

Table 22.1 Mean cell counts 15 minutes after injection of either αα-Hb or Hb-PEG510 (Enzon) into rats. Values are ± sem (n = 6 animals per group)

	Number of eosinophils/villus	Degranulated mast cells/villus	Number of villi
Saline	3.64 ± 0.33	0.65 ± 0.10	80
αα-Hb (US Army)	11.00 ± 0.56	1.33 ± 0.26	60
Hb-PEG5K10 (Enzon)	7.54 ± 0.39	2.27 ± 0.26	70

Both LEH and liposomes activated human complement, and the effect was primarily due to the presence of the phospholipid vehicle. Soluble complement receptor type 1 efficiently inhibited all vesicle-induced complement activation. The HBOC itself, in this case bovine stroma-free tetrameric Hb from Biopure Corporation (Cambridge, MA), did not activate complement, similar to the results *in vivo* with αα-Hb.

MICROVESSELS AND INTESTINAL MUCOSA

It is well known that excess ROS oxidize lipids of the cell membranes. Lipid peroxidation damage of membrane components is thought to play an important role in increasing capillary permeability (Granger *et al.*, 1981). Since H_2O_2 can easily diffuse across cell membranes (Henderson and Chappell, 1993), and O_2^- can traverse membranes via the chloride anion channel (Terada, 1996), it is likely that these reactive oxygen species will leave the microvasculature and gain access to other cells in the tissue. This action can have deleterious effects. For example, if the ROS reach mast cells in the interstitium they will cause the mast cells to degranulate (Kubes *et al.*, 1993) and release a selection of inflammatory mediators, including histamine. Many studies show that histamine increases microvascular permeability (Fox *et al.*, 1980; Horan *et al.*, 1986; Wu and Baldwin, 1992; Baldwin and Thurston, 1995; Thurston *et al.*, 1995). In addition, degranulating mast cells release eosinophil and neutrophil activating factors, causing these cells to release more ROS. In normal blood, the reservoir of heme iron is compartmentalized within the erythrocyte, which limits its ability to act as a catalyst. Erythrocytes are rich in antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, which inactivate the O_2^- and H_2O_2, respectively. These enzymes are in close proximity to Hb within the erythrocytes, and although Hb can release catalytic iron when exposed to oxidant stress, injury is limited because the enzymes react with the resultant ROS before they reach the cell membrane. This feature of erythrocytes was not considered in the design of the HBOCs that have been used so far in clinical trials. However, it is an important consideration because formation of the unstable form of hemoglobin, metHb, proceeds rapidly after injection of PolyBvHb (Lee *et al.*, 1995). In fact, D'Agnillo and Chang (1993) developed a crosslinked hemoglobin linked to superoxide dismutase and catalase which they showed could scavenge oxygen-derived free-radicals and thus prevent methemoglobin formation and iron release (see Chapter 18).

Although injection of HBOCs quickly produces excess ROS, very few experiments have been performed to determine whether injection of HBOCs damages microvessels or epithelial surfaces. One study showed that bolus injection of αα-Hb increased venular leakage to bovine serum albumin (BSA) and produced mast cell degranulation in the rat mesentery (Baldwin, 1999), while two other studies reported detachment of intestinal epithelial cells from each other, and from the basement membrane (Baldwin, 1997; Baldwin *et al.*, 1998). Such changes are characteristic of an inflammatory response (Baldwin and Thurston, 1995; Wilson and Baldwin, 1999). This is not surprising because mast cell degranulation, as observed after injection of αα-Hb, causes release of histamine and other inflammatory mediators, which then damage cell membranes. It is disadvantageous for a potential blood substitute to cause microvascular leakage, because the substitute itself will rapidly leave the circulation and in addition alterations in transvascular exchange of plasma proteins will disturb the fluid balance between blood and tissue. Increased microvascular leakage also changes the kinetics of delivery of intravascularly injected drugs, and of endogenous enzymes and hormones, to various tissues. When transfusions are needed, for example after hemorrhagic shock, it is important that regulation of microvascular exchange is not compromised.

In another study, it was shown that mesenteric microvascular leakage to BSA, mesenteric mast cell degranulation, and epithelial disruption were all significantly lower in animals treated with PolyHbBv (Biopure Corporation, Cambridge, MA) compared to αα-Hb (Baldwin *et al.*, 2002a). This finding is consistent with the fact that injection of PolyHbBv results in a lower production of excess ROS in the intestinal mucosa compared to injection of αα-Hb. However, even in animals treated with PolyHbBv, the average number of leaks per unit length of venule was still significantly greater than for controls that were perfused with Hepes buffered saline. It is possible that PolyHbBv causes less damage than αα-Hb because PolyHbBv is more resistant to irreversible oxidative processes, which include the formation of long-lived ferryl species and subsequent heme degradation and iron loss (Nagababu *et al.*, 2002).

In contrast to the experiments previously described demonstrating that perfusion with modified hemoglobin increases microvascular permeability, another study showed that hypervolemic infusion and isovolemic exchange transfusion (50 per cent) of hamsters with diaspirin crosslinked hemoglobin did not enhance microvascular leukocyte–endothelium interaction or endothelial permeability (Nolte *et al.*, 1997). However, in these experiments the fluorescent tracer molecule that was used to assess microvascular leakage, FITC-dextran, had a molecular weight of 150 kDa, whereas the tracers used in the other studies were smaller (Smith *et al.*, 1989; Seibert *et al.*, 1991; Baldwin *et al.*, 2002a). Thus, it is possible that FITC-dextran was too large to extravasate through the endothelial gaps produced by hemoglobin. However, a more likely explanation for the discrepancy concerns the method by which microvascular leakage was assessed. In this study the extravasated FITC-dextran was quantified as the quotient of average fluorescence intensity outside the vessel versus that inside the vessel segment. In other studies it was demonstrated that macromolecular extravasation from the microcirculation in response to modified hemoglobins and to inflammatory mediators occurs in very discrete sites along the venules (Fox *et al.*, 1980; Wu and Baldwin, 1992; Baldwin and Thurston, 1995; Thurston *et al.*, 1995; Baldwin *et al.*, 2002a). Therefore it is possible that these small discrete areas of venular leakage were missed in the measurements of the average fluorescence intensity outside each vessel segment.

CLINICAL IMPLICATIONS OF INFLAMMATION DURING USE OF BLOOD SUBSTITUTES

The clinical implications of the effects of HBOCs, other than those relating to oxygen delivery, are not always recognized. For example, in a recent report of a clinical trial to test HBOC-201 (Hemopure™; Biopure Corporation, Cambridge, MA), the authors, on finding that the mean concentration of metHb in the patients was 7 per cent following infusion with HBOC-201, concluded that this concentration would probably have little clinical significance because it did not significantly alter the delivery of oxygen to organs (Sprung *et al.*, 2002). However, metHb will result in production of excess ROS, such as H_2O_2 and O_2^-, and this could be fatal in patients with existing inflammatory conditions, such as ulcerative colitis. For example, H_2O_2 and O_2^-, together with chelate iron from denatured Hb, amplify the inflammatory response and subsequent mucosal damage in such patients (Babbs, 1992). Neutrophils, macrophages, eosinophils and mast cells that are activated by excess ROS produce more oxygen metabolites that may be toxic to erythrocytes, endothelial cells and fibroblasts. The resulting lysis of erythrocytes leads to the release of free Hb into the blood. If activated neutrophils are in the area, their associated enzymes will enhance the oxidation of the free Hb to metHb, promoting further production of excess ROS. In addition the chemotactic products of lipid peroxidation provide positive feedback to accelerate the inflammatory process. The resultant oxidative stress leads to extension and propagation of crypt abscesses. It is possible that the combination of ulcerative colitis and transfusion with an HBOC could result in destruction of the endothelial/epithelial barrier between the intestine and the bloodstream, possibly leading to septicemia.

Approximately one million Americans suffer from ulcerative colitis, and there are about five new cases per 100 000 individuals per year (Babbs, 1992). Since ulcerative colitis is a significant disease of young adults who are otherwise healthy, it is possible that this condition could be missed in such people requiring a rapid transfusion. Thus it is essential that patients be thoroughly checked before involving them in clinical trials of the Hb-based blood substitutes that are currently being developed.

ANTICIPATED FUTURE DIRECTIONS

It is obvious that in order to prevent oxidative tissue damage by hemoglobin-based blood substitutes during transfusion, the formation of and the effects of different nitrogen- and oxygen-derived radicals must be prevented. To prevent formation of such radicals it is necessary to reduce the tendency of the hemoglobin to oxidize by chemical modification during manufacture. Another way to prevent Hb-induced tissue damage is to use an iron chelator. So far this approach has not been used clinically, but animal experiments have shown that microvascular leakage produced by bolus injection of $\alpha\alpha$-Hb can be ameliorated by concurrent infusion of an iron chelator, C-10 phosphorothioate

oligodeoxynucleotide (PS-ODN), developed by AVI BioPharma, Corvallis, OR (Baldwin *et al.*, 2002b). The product has been shown to have a high affinity for iron, and facilitates iron excretion (Mata *et al.*, 2000). PS-ODN has the advantage over the more widely used chelator, desferrioxamine, because treatment with desferrioxamine is costly, requires parenteral administration, and has side effects associated with its use. However, systemic administration of C-10 PS-ODN did not prevent αα-Hb-induced damage to the epithelium of the intestinal mucosa, but made it worse (Baldwin *et al.*, 2002c), indicating that some types of hemoglobin-induced tissue injury may occur by means other than release of free iron. Spectrophotometric measurements of αα-Hb oxidation *in vitro* indicate that the presence of PS-ODN may increase the transformation of αα-Hb to its ferryl form. Since αα-Hb-induced epithelial damage was worsened in the presence of PS-ODN, this indicates that the epithelium may be particularly vulnerable to ferryl Hb. Several direct strategies are emerging aimed at cycling ferryl back to ferric hemes by stimulating a catalase-like activity of hemoproteins using nitroxide (Krishna *et al.*, 1996) or the addition of Trolox™, a vitamin E analog known for its anti-ferryl activity (Giulivi *et al.*, 1992).

Alternatively, oxidants could be scavenged by using antioxidants, such as catalase and superoxide dismutase (SOD), which are endogenous scavengers for H_2O_2 and O_2^-, respectively. Several methods currently incorporate SOD and catalase into crosslinked Hb (e.g. D'Agnillo and Chang 1993). This technique ensures that the free-radical scavengers are in close contact with the source of the ROS, the hemoglobin, and also effectively addresses the problem that SOD has a short half-life in blood (10–40 minutes). An alternative way of increasing the half-lives of SOD and catalase is to bind them to PEG. Polyethylene glycol-SOD has a half-life of several days, and PEG linkage increases the half-life of catalase from 2 to 50 hours.

Another antioxidant that has been investigated for use with blood substitutes is nitroxide. Nitroxides are able to scavenge O_2^-, and thus can act as potent antioxidants. However, free nitroxide is cleared very rapidly from the circulation and very little is left even 5 minutes after intravenous infusion. For this reason a polynitroxylated hemoglobin-based oxygen carrier has been developed in which nitroxide molecules are covalently bound to Hb so that the circulatory half-life of the nitroxide molecules is greatly increased (Saetzler *et al.*, 1999). At this time there is no published record of the ability of hemoglobins, conjugated with antioxidants, to minimize oxidative tissue damage.

Ideally, a therapeutic agent would scavenge all deleterious radicals, whether or not their production is catalyzed by iron, and would be able to cross biological membranes. One possible candidate that has been suggested is Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N oxyl), a stable nitroxide that attenuates the effects of peroxynitrite and oxygen-derived free radicals such as superoxide anions and hydroxyl radicals (Thiemermann *et al.*, 2001). Unlike recombinant superoxide dismutase, which is not able to cross biological membranes, Tempol permeates biological membranes and accumulates in the cell cytosol. Future studies using molecules such as Tempol, in conjunction with a range of Hb-based blood substitutes with various oxygen affinities and nitric oxide scavenging capacities, may lead to a product that is both non-toxic and delivers oxygen at a rate suitable for the conditions.

If such an ideal product cannot be developed, it might be possible to manipulate the endothelial cells such that they can protect themselves from oxidative damage. If the endothelial cells become damaged, this will lead to the influx of unstable Hb-derived products into the surrounding tissue, and so it is critical that the endothelium remains intact. Previous experiments (Abraham, 1995) on cultured endothelial cells have shown that when these cells are transfected with a gene encoding the enzyme, heme oxygenase, their survival rates after exposure to recombinant human Hb (rHb, Somatogen, Boulder, CO) increased from 70 per cent to 95 per cent. Perhaps in the future, pharmacological manipulation of heme oxygenase to enhance its synthesis and activity could be used to offer cellular protection against Hb oxidative injury.

SUMMARY

There is some experimental evidence to support the idea that modified hemoglobins, injected *in vivo*, oxidize to form reactive oxygen species (ROS). Although cell-free Hb may present a low risk to people with normal redox status, patients who are sick and have a poor antioxidant supply may be at risk because excess ROS can cause

tissue damage and organ dysfunction. Tissue damage may occur by formation of the highly reactive ferryl Hb, by formation of the hydroxyl radical by the Fenton reaction in which free iron acts as a catalyst, or as a result of reactions of nitric oxide with ROS or with Hb. Animal studies have shown that bolus injection of a variety of HBOCs stimulates an inflammatory response in the intestinal mucosa, as evidenced by mast cell degranulation, recruitment of eosinophils, epithelial disruption and microvascular leakage. However, the clinical implications of the effects of HBOCs, other than those relating to oxygen delivery, are rarely acknowledged. For example, the combination of ulcerative colitis and transfusion with an HBOC could result in destruction of the endothelial–epithelial barrier between the intestine and the bloodstream, possibly leading to septicemia. In addition, future complications, such as cancer, could be triggered in these patients by HBOC infusion. In order to prevent oxidative tissue damage by hemoglobin-based blood substitutes during transfusion, the formation of and the effects of different nitrogen- and oxygen-derived radicals must be prevented either by reducing the tendency of the hemoglobin to oxidize by chemical modification during manufacture or by incorporating the most effective antioxidants in the transfusion or in the HBOC itself. Alternatively, methods could be developed to manipulate the enzyme, heme oxygenase, pharmacologically in order to enhance its synthesis and activity so it could provide protection to endothelial cells against Hb oxidative injury.

EDITOR'S SUMMARY

Redox cycling of iron contained within hemoglobin, regardless how the hemoglobin is modified, remains a concern because such cycling can lead to reactive oxygen species (ROS) that can cause tissue damage. If hemoglobin extravasates or penetrates endothelial or parenchymal cells, such ROS could be even more destructive. Steps taken to minimize such effects include encapsulation of hemoglobin within liposomes or nanocapsules, or copolymerization of hemoglobin with antioxidant proteins. While this mechanism could be very important in explaining some of the toxicities seen in some human trials, the nature of human trials precludes establishing links between biochemical mechanisms and human pathology. The gastrointestinal tract is an especially relevant model to study these effects; because of redistribution of blood flow in shock, the GI tract can become relatively ischemic and susceptible to 'reperfusion' injury when blood flow is restored.

This chapter describes studies of the GI tract in animals that have been given various hemoglobin-based oxygen carriers. Clear evidence is presented for penetration of tissue by these products, as well as stimulation of inflammatory cells, histamine release and complement activation. The studies warn that while some products might appear to be safe, based on studies in normal animals or even in normal volunteers or stable surgical patients, more severe and undesirable effects could accompany administration to patients in hypovolemic shock – precisely the population targeted by developers of most products. It is hoped that as more products become available for general experimentation, these studies can be extended enough to allow generalizations as to which modifications are more dangerous, and which might ameliorate the effects of ROS.

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Chapter 23

Hemoglobin-Induced Myocardial Lesions

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INTRODUCTION

Considerable progress has been made over the past 20 years to develop a safe and effective hemoglobin-based oxygen carrier (HBOC) for clinical use. Many of the issues encountered in the very early attempts to develop these products have been addressed with the HBOC products currently being tested. Early 'stroma reduced' hemoglobin solutions had four primary safety concerns: short intravascular half-life, renal toxicity, residual red cell stroma, and endotoxin contamination. These issues have been resolved by stabilizing the hemoglobin tetramer using chemical and recombinant means, enhancing the size of the hemoglobin product using polymerization and/or derivatization technologies, and using advanced techniques of protein purification and process engineering.

More recently, it has become evident that native hemoglobin interacts very strongly with nitric oxide (NO), a ubiquitous and potent chemical messenger found throughout the body. After review of a number of clinical trial results and additional preclinical study results, it appears that the interaction of hemoglobin with NO, and the physiologic and pathophysiologic consequences of this interaction, may be responsible for many of the adverse effects observed with the first generation of purified and modified hemoglobin solutions that were investigated in the clinic in the 1990s (hereafter referred to as first-generation HBOCs). The following represents a list of 'potential' issues that have been associated with the first generation HBOCs:

- vasoactivity/increase in arterial pressure
- extravasation of hemoglobin
- relatively short half-life
- increase in serum enzymes
- effects on the pancreas
- gastrointestinal dysmotility
- oxidant stress
- Hb/endotoxin interaction
- cardiac lesions.

Of these potential issues, the presence of myocardial lesions represents an important histopathologic finding that must be considered during the preclinical testing and development of new HBOCs.

BACKGROUND

Diaspirin crosslinked hemoglobin (DCLHb, Baxter Healthcare Corp.) is a first-generation modified human hemoglobin solution produced by reacting deoxygenated human hemoglobin with the crosslinking agent bis(3,5-dibromosalicyl)

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fumarate (DBBF) to form a stabilized tetramer that is covalently linked between the alpha globin chains (Walder *et al.,* 1979; Chatterjee *et al.*, 1986). During the initial toxicological evaluations of DCLHb, single-dose studies were performed in several standard animal species. In studies in rats and dogs, no adverse effects of DCLHb on the heart were observed with doses up to 40 ml/kg (4000 mg DCLHb/kg). Dose escalation and repeat dosing studies with DCLHb were subsequently conducted in cynomolgus monkeys. During the microscopic examination of heart tissues in these studies, myocardial degeneration and/or necrosis of mild to moderate severity was observed in animals treated with moderate to high doses of DCLHb.

After discovery of these lesions, a variety of different experiments were performed to better understand their etiology, pathogenesis and clinical significance. The initial objective of this work was to develop a relevant, sensitive and reproducible animal screening model in which heart lesions similar to those seen in primates could be produced in response to hemoglobin administration. Another objective was to characterize fully the myocardial lesions in those species in which this pathology was observed. Finally, the mechanism of lesion development was studied and interactions designed to mitigate lesion development were assessed.

ANIMAL MODELS

Although originally found in cynomolgus monkeys and subsequently observed in African green monkeys, both of these species were significantly less sensitive than rhesus monkeys to the development of myocardial lesions (Figure 23.1). Cynomolgus monkeys infused with 2000 mg/kg of DCLHb typically developed lesions of myocardial degeneration and/or necrosis graded as minimal in severity (1.0 on a scale of 0–4) with an incidence of 67 per cent. In contrast, rhesus monkeys developed more severe heart lesions at relatively low doses of DCLHb.

While these data demonstrate that primates are very sensitive to the development of this lesion, the use of primates for screening purposes was not practical. Therefore, experiments were performed to identify a more cost-effective model that would allow rapid and reproducible screening for identifying potential mechanisms and/or co-medicaments with the least number of

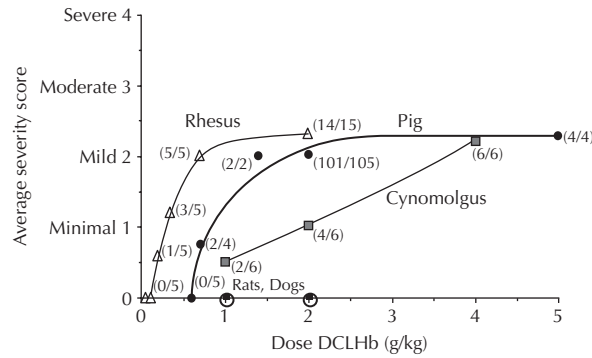

Figure 23.1 Incidence and dose–response of myocardial degeneration and necrosis in various species. Note: The number included in parentheses reflects the incidence/number of animals examined.

animal use issues. This goal was challenging, since heart lesions had not been observed in experiments performed with dogs, sheep or rats following single infusions of DCLHb, implying that the no-effects doses in these species were greater than 4000 mg/kg.

Heart lesions with a similar appearance could be produced in rabbits; however, ten-fold higher doses of DCLHb (>3000 mg/kg) were required to produce a comparable incidence and severity to that seen in rhesus monkeys. Moreover, in rabbits there was a significant background incidence of degenerative, necrotic and inflammatory heart lesions in untreated, sham-treated, and control rabbits.

On the other hand, it was found that swine were a very good model because they consistently developed heart lesions after infusion of moderate doses of hemoglobin solutions with a low level of background incidence. In addition, swine are generally recognized as a good species for studying the effects of agents on the cardiovascular system, and are also a species that reproduces the hemodynamic responses observed in humans after the infusion of DCLHb with respect to increases in mean arterial blood pressure. Therefore, it was decided to further develop the swine model for cardiac lesion development. With a no-effects dose less than 700 mg/kg, swine appeared to be more sensitive than cynomolgus monkeys and almost as sensitive as rhesus monkeys (Figure 23.1).

To ensure comparability between experiments, the swine model used for evaluation of heart lesions was standardized. All experiments utilized young crossbred domestic swine that weighed

between 10 and 20 kg. Approximately 24 hours prior to dosing, animals were anaesthetized and chronic catheters were placed in the jugular vein, and sometimes in the carotid or femoral artery, for infusion of test and control articles and blood sampling for clinical chemistry analysis. Test or control solutions were infused intravenously using an infusion pump at a constant rate of 1 ml/kg per minute. When assessing the effect of interventions, a standardized DCLHb dose of 2000 mg/kg was typically infused. This dose was found to be the best compromise between minimizing volume load and consistently producing lesions. As a treated control, human serum albumin (HSA) that was oncotically matched to each test article was infused into separate swine at the same rate and volume. Blood samples were routinely taken at baseline, immediately post-infusion, and at 8, 24 and 48 hours post-infusion of test or control article for the measurement of various enzyme levels. Clinical observations were performed throughout the experiments. At approximately 48 hours post-dosing, the animals were euthanized, a complete necropsy examination was performed, and various tissues (including the heart) were taken for histopathologic evaluation. The heart specimens routinely examined consisted of the atria, left and right ventricular free walls, and inter-ventricular septum, including associated papillary muscle. As noted in more detail below, 48 hours post-dosing was determined to be the optimal time for sacrifice because this interval allowed enough time for the lesion to develop, yet was not so long after the initial insult that damaged myocardial cells might already be removed and cleared by repair processes in the body.

LESION CHARACTERISTICS

Once sensitive and reproducible animal models were identified, the hemoglobin-induced myocardial lesions were characterized in more detail. In primates, myocardial lesions observed after hemoglobin infusion consist of a minimal to moderate myocardial degeneration characterized by cytoplasmic swelling and vacuolization of myofibers, occurring primarily in the left ventricle and/or septum. The lesions are usually focal or multifocal in distribution, sometimes only involving a few cells, although occasionally they are locally extensive. Often the degeneration is associated with foci of coagulative myofiber necrosis that display a homogeneity to granular eosinophilic staining cytoplasm. Enlargement of the nuclei (karyomegaly) of myocytes and minimal to mild interstitial fibrosis are also frequently associated with the degenerative lesions. The karyomegaly is interpreted as a reactive change. In some animals, a mild lymphocytic inflammatory infiltrate is also present.

Similar to the lesions observed in primates, the lesions found in swine were described as myocardial degeneration and/or necrosis, with a focal to multifocal distribution, and of minimal to moderate severity. The myocardial degeneration was characterized by focal cytoplasmic swelling and slight hypereosinophilia of myofibers, whereas necrosis was evident as areas of moderate to marked homogeneity to granular eosinophilic cytoplasmic staining with shrinkage (pyknosis), fragmentation (karyorrhexis) or lysis (karyolysis) of the nuclei. Necrotic areas were usually associated with a mononuclear inflammatory infiltrate consisting of macrophages and a lesser number of lymphocytes. Mineralization of cellular debris could also be observed occasionally at some sites of necrosis. Figure 23.2 shows microscopic

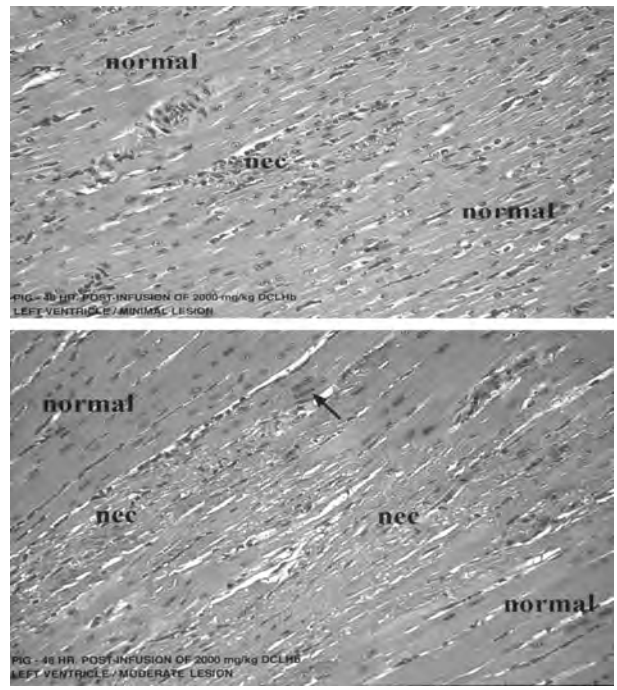

Figure 23.2 Photomicrographs of H&E stained sections of myocardium from the left ventricle of different swine following infusion of 2000 mg/kg of DCLHb, illustrating typical heart lesions of different severity (nec = necrosis).

changes involving the left ventricle of swine, illustrating typical lesions of necrosis classified as minimal or moderate following a single intravenous infusion of 2000 mg/kg DCLHb.

The myocardial changes observed after hemoglobin infusion are very similar in appearance to those seen after the administration of high doses of sympathomimetic amines such as epinephrine, norepinephrine and dopamine (Haft, 1974). For example, infusion of dopamine into dogs during a 14-day subacute toxicity study resulted in the formation of myocardial lesions (FDA Summary Basis of Approval, 1973). However, the fact that catecholamine depletion did not mitigate the formation of heart lesions in swine after subsequent hemoglobin infusion suggests that this pathway is not involved in the hemoglobin induced response. Several experts have also noted that the lesions appear different in their histological appearance, time-course, and severity than those seen after infusion of other cardiotoxic drugs, such as adriamycin (Buja *et al*., 1973, 1974a, 1974b).

To quantify the characteristics of the cardiac lesions using anatomic pathology, both incidence and severity parameters were utilized. Incidence was defined as the number of hearts that exhibited any evidence of lesion formation divided by the total number of hearts examined (e.g. 2/4). Severity was a measure of lesion intensity and extent that was scored by the evaluating pathologist on an ascending scale of 0–4. Grade 1 lesions were considered minimal, Grade 2 lesions mild, Grade 3 lesions moderate, and Grade 4 lesions severe. In a given group of tissue specimens, an overall average severity score was calculated by summing the severity grades for each affected heart and dividing by the total number of hearts evaluated in that group.

By combining data from several studies, the variation of lesion incidence and severity after the administration of single doses of DCLHb was defined in rhesus monkeys (Table 23.1). Above the no-effect level of 100 mg/kg a dose–response relationship was observed, with a 100 per cent incidence and maximization of the average lesion severity at a score of approximately 2.3 at 700 mg/kg. At substantially higher doses, no significant increase in the severity of the lesion was observed.

The 'self-limiting' nature of the severity of the lesion is apparent in the data accumulated in a 28-day repeat dose toxicity study in rhesus monkeys. This study investigated the toxicity of DCLHb in awake rhesus monkeys following daily infusions of DCLHb for 28 days, with doses ranging from 1000 to 4000 mg/kg per day. The planned sacrifice intervals were either Day 29 (after receiving 28 doses) or Day 64, with the latter providing 28 days of daily dosing followed by a recovery period. Although some animals received cumulative doses as large as 112 000 mg/kg of DCLHb, the severity of the lesions was no greater (average severity score of 2) than that seen in the earlier single-dose studies. Even more intriguing was the observation that many animals had no histologic evidence of myocardial lesions following infusion of DCLHb at cumulative doses substantially greater than the 100 mg/kg no-effects dose in single-dose studies. For example, only three of seven monkeys dosed with 2000 mg DCLHb per kg per day and examined at Day 29 had histological evidence of myocardial lesions, despite the fact that they each received a cumulative dose of 56 000 mg/kg of hemoglobin. This is in direct contrast to the results in monkeys that received a single 2000-mg/kg infusion (Table 23.1), in which the incidence of heart lesions was 14/15 monkeys. The explanation for this difference in response is not known with certainty, but may reflect the competency of the cardiac tissue repair process.

To assess the time course of lesion development, tissues collected from animals sacrificed at different time intervals were examined microscopically. From these examinations, it was concluded that the degenerative myocardial changes appeared as early as 1–6 hours post-infusion. Electron microscopy was required to detect the changes at early time points. Lesions are detectable by light microscopy from approximately 24 to 72 hours after hemoglobin administration. Evaluation of tissues at earlier or later time intervals by light microscopy may not detect the presence of this pathology. While some degenerative cells became necrotic, others apparently

Table 23.1 Incidence and severity of heart lesions in primates

Dose (mg/kg)	Incidence	Average severity score
50	0/5	0
100	0/5	0
200	1/5	0.6
350	3/5	1.2
700	5/5	2.0
2000	14/15	2.3

recovered their normal appearance and function. Necrotic tissue was ultimately removed and subsequently replaced, in part, by fibrous connective tissue. Another component of the recovery process was the enlargement of myocytes adjacent to affected areas, which probably represented a physiologic hypertrophy caused by increased functional demand on the unaffected cells. Morphologic evidence of muscle fiber regeneration was also evident in swine. The long-term consequence of myocardial lesion development was the loss (necrosis) of a small fraction of the myocytes originally present, which were replaced by proliferation of connective tissue and possible regeneration of muscle cells.

To examine the severity of the lesions quantitatively, a morphometry study was conducted in rhesus monkeys after the infusion of 2000 mg/kg of DCLHb. In this study, five animals were infused intravenously with 20 ml/kg of a 10 g DCLHb solution at a rate of 1.0 ml/kg per minute, the animals were sacrificed 7 days post-infusion, and the heart tissue was collected, fixed, sectioned and examined by morphometric analysis. Myocardial lesions were observed in four out of five monkeys. In the affected hearts, the mean fraction of tissue with degeneration or necrosis was 1.01 per cent (range = 0–2.92 per cent). The most sensitive tissue was the left ventricular papillary muscle (mean = 0.89 per cent; range = 0–2.72 per cent), followed by the left ventricular free wall and interventricular septum (mean = 0.11 per cent; range = 0–0.36 per cent). The right ventricle was sometimes affected (mean = 0.01 per cent; range 0–0.04 per cent), but to a much lesser degree. The atria were almost never affected (0 per cent).

In this experiment, as well as in many subsequent experiments in a number of different animal species, an attempt was made to identify a clinical pathology marker for myocardial injury that would allow for more rapid and sequential monitoring of lesion development. Unfortunately, to date no statistically significant increases in typical markers of myocardial injury, such as the myocardial isoenzyme of creatine kinase (CK-MB) or the lactic dehydrogenase isoenzyme LDH-1, were observed following infusion of large and repeated doses of DCLHb, even in sensitive species such as the rhesus monkey. The small percentage of myocardium involved in the monkeys is consistent with the observation that no significant levels of cardiac specific enzymes were identified in the plasma after hemoglobin infusion. In fact, to date no surrogate marker of myocardial injury has been identified in any animal species. Likewise, utilizing electrocardiography (ECG), no functional consequences of cardiac lesion development could be detected.

MECHANISM OF HEMOGLOBIN-INDUCED MYOCARDIAL LESIONS

Over the past decade there has been a substantial effort to understand the mechanism of heart lesion formation following the intravenous infusion of hemoglobin solutions (Burhop and Estep, 2001). Likewise, using the standardized swine model, possible methods for mitigation of this process were extensively examined. Efforts were primarily directed to the testing of a large number of compounds whose mechanism of action might be hypothesized to interfere with a putative mechanistic pathway. In considering these experiments, it should be noted that the standardized DCLHb dose of 2000 mg/kg produced heart lesions in 96 per cent of the treated animals (i.e., 101/105 animals had lesions) with an average severity score of 2.1. Occasionally lesions were seen in animals that were infused with HSA, but the incidence and severity was extremely low (i.e., 3/27, or 11 per cent, of the animals infused with 1600 mg/kg of HSA had lesions with an average severity score of 0.1). Background lesions were not routinely seen in normal, untreated swine (i.e., 0/17 sham animals had lesions). For the purposes of this overview, experimental results will be summarized. The primary endpoint in each case was histologic evaluation of the hearts as quantified by the myocardial lesion incidence and overall severity score.

Contaminants

Infusion of DCLHb that was subjected to an additional chromatographic purification step produced the same results as the standard DCLHb solution (i.e., 4/4 animals had lesions with an average severity of 2.5), suggesting that contamination was not responsible for causing the lesion. Moreover, the fact that heart lesion development was observed after the administration of either human or swine stroma-free hemoglobin, or a recombinant hemoglobin produced by bacterial fermentation, strongly argues that the finding is attributable to hemoglobin rather than a contaminant, since the contaminant profiles of these preparations is expected to be very different.

Effect of a foreign protein

Infusion of purified, uncrosslinked, human stroma-free hemoglobin (SFH) produced the same heart lesion as DCLHb with the same incidence (4/4), albeit with a slightly reduced severity (mean severity of 1.3). The reduced severity was probably due to the considerably shorter circulating half-life of unmodified hemoglobin compared to DCLHb, due to the rapid excretion of the SFH through the kidney. This would be expected to somewhat reduce the direct exposure of the heart to the SFH. Infusion of purified swine SFH into pigs caused the same heart lesion as that seen following infusion of DCLHb or human SFH (i.e., incidence of 5/6 animals with a mean lesion severity of 2.3), suggesting that this phenomenon is probably a more general property of acellular hemoglobins. These experiments also demonstrated that myocardial lesions were not due to infusion of a human protein into a non-human species.

Heme

In order to investigate whether heart lesion formation could be related to the reduced heme component of DCLHb, the effect of conversion of the heme to the cyanomet form was examined. In these experiments, the met-DCLHb was prepared by addition of potassium ferricyanide to oxidize the hemoglobin. The excess potassium ferricyanide was removed, and potassium cyanide was added in slight excess. It was found that conversion to the cyanomet form had no significant effect on the incidence and/or severity of the heart lesion (i.e., incidence = 3/4 with a mean severity of 1.3), although this result may have been compromised by *in vivo* conversion of cyanomet Hb to reduced Hb.

This conversion to reduced Hb, although surprising, can be explained by the following rationale: In an equilibrium, static solution, only a slight excess of cyanide would be needed to saturate the oxidized hemoglobin fully, because cyanide is so tightly bound. However, the live animal is not a static solution and there are reducing agents present, such as glutathione. In addition, there are other sinks for CN to be bound (such as other cytochromes and oxidized red cell hemoglobin) competing with the oxidized DCLHb for cyanide binding. Therefore, the cyanomet DCLHb when infused in living animals is in a dynamic environment with reducing equivalents present. Antonini and Brunori's laboratory showed a number of years ago that in the presence of a reducing agent (dithionite), CN dissociates from various Hb and Mb variants with a half-life of a few seconds at pH 7 (Bellelli *et al.*, 1990, 1994). In other words, the dissociation rate of CN from met-DCLHb is much faster than the half-life of DCLHb elimination from the body. Therefore, in the animal infused with cyanomet-DCLHb, the CN would dissociate from met-DCLHb and the met-DLCHb would be reduced during the time when DCLHb was circulating in the vasculature.

CO-MEDICAMENTS

To gain further insight into the potential mechanism of heart lesion formation, as well as to identify potential interventions that would be clinically useful, the effect of co-administration of many different agents with varying pharmacologic actions was assessed. In the typical experiment, the standardized swine testing protocol was utilized, with the key independent variable being the co-medicament. In some cases, a variety of dosing regimens or administration protocols were evaluated with each agent. Co-medicaments examined included a variety of antihypertensives, such as nicardipine, adenosine, phenoxybenzamine, propranolol, verapamil, captopril, ATP-$MgCl_2$, metroprolol, halothane, sodium nitroprusside and L-arginine. Several different anticoagulants, such as aspirin, dipyridamole and heparin, were examined, as were several different anti-inflammatory agents, such as dexamethasone, ibuprofen and benadryl. A broad spectrum of antioxidants, including taurine, vitamin E, selenium, ascorbate, OTC (L-2-oxothizolidine-4-carboxylic acid), MPG (N-2-mercaptopropionyl glycine), oxypurinol, mannitol, lactobionate, carnitine, allopurinol and lipoic acid were also tested. Finally, the iron-binding agent deferoxamine was also evaluated. After extensive testing, no effective co-medicament was identified; nor was any definitive mechanism of action elucidated in this extensive series of experiments.

Administration protocol

The impact of several different variations in the hemoglobin administration protocol was evaluated. For example, the hemoglobin was administered via a topload (hypervolemic) infusion; via

differing levels of isovolemic exchange transfusion; pre-dosing with haemoglobin was tested; dosing of hemoglobin in hemorrhage/resuscitation protocols was evaluated; different animal sources were evaluated; the effect of animal gender was evaluated; the effect of splenectomy, hydration state, or anesthesia were tested; and finally, the effect of catecholamine depletion before hemoglobin administration was evaluated. In summary, the administration protocol utilized in the testing did not seem critical, as similar lesions were observed when the hemoglobin was administered as a volume load, by exchange/transfusion, or to hemorrhaged animals, suggesting that the phenomenon is not due to volume overloading.

Chemical modification

To assess the potential effect of polymerization and the molecular size of the hemoglobin molecule on the generation of heart lesions, several experiments were performed with different DCLHb derivatives. In one study, DCLHb was treated with gluteraldehyde to create a polydisperse family of hemoglobin polymers. This solution was then diafiltered against a membrane having a nominal 300 000-Dalton molecular weight cut-off. The resulting retentate solution was essentially free of unpolymerized hemoglobin tetramers, while the filtrate was enriched in this molecular weight fraction. After diafiltration into the same electrolyte vehicle, these two solutions were infused into swine. The lesion incidence and overall severity scores were lower in animals that received the polymerized DCLHb retentate (2/5 and 0.5, respectively) compared to those animals treated with filtrate (5/5, 2.6). Similar results were obtained when DCLHb was polymerized with bifunctional polyethylene glycol-based reagents. In most cases both the incidence and severity of the heart lesions could be reduced, but not completely eliminated, by increasing the molecular size of the DCLHb. These data imply that the size of the hemoglobin molecule does have an influence on the generation of heart lesions, but that the lesions could not be completely eliminated in sensitive species simply by polymerization. It is believed that polymerization acts to reduce the rate of hemoglobin extravasation into heart tissue, and thereby lowers the hemoglobin concentration near sensitive cells.

Role of nitric oxide

As another part of the investigation into possible mechanisms of cardiac lesion development, the potential role of nitric oxide was investigated. Native hemoglobin interacts very strongly with nitric oxide (NO), a ubiquitous and potent chemical messenger found throughout the body. *In vivo*, nitric oxide scavenging by hemoglobin occurs primarily via two rapid reactions: the oxidative reaction of NO with oxyhemoglobin to produce nitrate and methemoglobin, and NO binding to deoxyhemoglobin to form a stable complex (Patel, 2000). Both reactions likely contribute to *in vivo* NO scavenging, with the relative significance depending on local abundances of oxy- and deoxyhemoglobin. There is also evidence that this scavenging of NO may be associated with some of the adverse outcomes observed with the first-generation hemoglobins. For example, studies in rats have clearly demonstrated that increases in mean arterial pressure observed immediately after hemoglobin infusion correlate directly with the rate of NO scavenging; as the NO scavenging is decreased, the pressor response is decreased (Doherty *et al.*, 1998). More recently, it has been reported that the chronic inhibition of nitric oxide production by L-NAME causes myocardial infarction in rats (Moreno *et al.*, 1997; Ono *et al.*, 1999). L-NAME is an inhibitor of the enzyme nitric oxide synthase that produces NO. Infusion of L-NAME at doses as low as 40 mg/kg into swine resulted in heart lesions similar in incidence (5/5), severity (mean = 1.5) and appearance to the lesions observed after infusion of DCLHb solutions.

To investigate the role of hemoglobin/NO interactions systematically, a series of genetically altered hemoglobins was produced using recombinant technology. These hemoglobins were specifically designed to exhibit varying rates of reaction with NO. Recombinant hemoglobins with NO scavenging properties similar to those of native human hemoglobin (e.g. rHb1.1 produced by Somatogen) produced heart lesions with the same incidence and severity as those seen with DCLHb. In contrast, recombinantly produced hemoglobin solutions that contained hemepocket modifications that reduced the rate of nitric oxide interaction exhibited a reduced rate of heart lesion formation after infusion into swine (Table 23.2). More specifically, a hemoglobin variant with a 25-fold decrease in nitric oxide reactivity produced no detectable heart lesions

Table 23.2 Incidence and severity of heart lesions in pigs infused with various hemoglobin solutions

Treatment agent	K'NO (μM/s)	MW (kD)	Incidence (%)	Overall severity
HSA	–	64	3/27 (11)	0.1
DCLHb	~60	64	35/37 (95)	1.9
rHb1.1	60	64	4/4 (100)	2.0
NO mutant Hb	2	64	0/4 (0)	0

in swine. This variant was internally crosslinked by recombinant techniques, and was very similar to rHb1.1 or DCLHb with respect to molecular weight, oxygen affinity and oxygen binding kinetics.

As a result of these promising results in swine, this same hemoglobin variant was subsequently tested in rhesus monkeys. In contrast to the results in swine, myocardial lesions were observed in all of the test animals following infusion into monkeys, although the lesion severity was substantially reduced. This led to exploration of the effect of the combination of polymerization and a reduced rate of NO interaction on heart lesion development. To do so, an intramolecularly crosslinked hemoglobin with reduced NO reactivity was polymerized and derivatized with a bifunctional polyethylene glycol reagent. This new material, designated as rHb2.0 for Injection, was evaluated in both single-dose and repeat-dose studies in rhesus monkeys. In a single-dose toxicity study in rhesus monkeys, no cardiac lesions were observed in animals that were sacrificed 48 hours after receiving a single dose of 500 (n = 8), 1000 (n = 8) or 2000 mg/kg (n = 8) of rHb2.0. In a separate group of monkeys that were sacrificed 2 weeks after dosing, there was also no evidence of myocardial lesions. In a repeat dose study in which rhesus monkeys received, every other day, infusions of either 1000 or 2000 mg/kg of rHb2.0 for Injection (10 animals per dose group) for a total of 7 infusions over 13 days, only one animal in the high-dose group exhibited a myocardial lesion, and it was focal and of minimal severity. Moreover, according to the reviewing pathologist, this lesion was of uncertain association with study drug administration since a background lesion of similar appearance is sometimes observed in monkeys. None of the other monkeys examined at the 48-hour sacrifice interval, or in the recovery group sacrificed 14 days after receiving the seventh dose, had any evidence of myocardial lesions. In total, only one of 56 monkeys receiving rHb2.0 for Injection exhibited any finding of myofiber degeneration or necrosis. These data suggest a major role for nitric oxide depletion in the mechanism of myocardial lesion development. Moreover, the combination of polymerization/derivatization and alterations to reduce the inherent rate of NO scavenging appear to be additive in that the lowest incidence of heart lesion development in rhesus was achieved with hemoglobin molecules that had both modifications.

SUMMARY

On the basis of all of the previous observations and pertinent facts from known data, a hypothesis can be generated for the mechanism by which hemoglobin induces the formation of cardiac lesions: It is known that hemoglobin scavenges nitric oxide. Experiments have been conducted that clearly demonstrate that infusion of nitric oxide inhibitors can produce myocardial lesions that appear very similar in appearance, location and severity to those produced by hemoglobin. It has been demonstrated that the papillary muscle of the left ventricle is the most sensitive myocardial tissue with respect to the adverse effects of hemoglobin infusion and nitric oxide inhibition, and it is known that the left ventricular papillary muscle may be one of the highest oxygen-consuming tissues in the body. There are numerous data demonstrating that infusion of hemoglobin into sensitive species, such as pigs and monkeys, produces significant increases in blood pressure, and thereby an increase in after-load on the heart. This increase in after-load on the heart likely results in increasing myocardial oxygen demand, which can result in a localized tissue hypoxia. As discussed earlier, polymerization of hemoglobin, which can slow down but not completely eliminate extravasation of hemoglobin from the vascular space, reduces both the severity and incidence of the myocardial lesions. Finally, recent data suggest that inhibition of nitric oxide synthesis increases mitochondrial oxygen consumption and may also affect Ca^{2+} hemostasis (Shen *et al.*, 1994; Bernstein *et al.*, 1996; Arstall and Kelly, 1999; Henry and Guissani, 1999; Zhao *et al.*, 1999; Boveris *et al.*, 2000).

When considered as a whole, these facts suggest that infusion of hemoglobin leads to enhanced oxygen consumption throughout the body

as a consequence of a reduction in tissue levels of nitric oxide. In the heart, especially in the papillary muscle, there is an increase in mitochondrial oxygen consumption due not only to the decrease in NO levels, but also as a consequence of the increased after-load due to peripheral vasoconstriction. As a result, oxygen demand may exceed oxygen supply in the most sensitive cells in the heart, leading to microscopic areas of hypoxia, cell injury and, ultimately, death. Likewise, as a result of interactions between hemoglobin and NO, there may be alterations in calcium hemostasis that may ultimately lead to myocardial cell degeneration and necrosis (i.e., produce contraction band necrosis). The inflammatory response that is seen in conjunction with the necrosis is likely a secondary event that represents removal, by macrophages, of necrotic myocardial cells.

It is important to note that to date no evidence of a hemoglobin-induced myocardial lesion has been observed in man. Furthermore, there have been no increases seen in enzymatic markers of myocardial injury such as CK-MB or troponin-I in any of the human clinical trials that have been conducted with DCLHb. However, the detection of hemoglobin-induced heart lesions in humans is confounded by the fact that patients treated with hemoglobin therapeutics likely have myocardial damage from other causes. It is therefore unclear whether the lesions observed in swine or primates occur in man. Nevertheless, the presence of myocardial lesions represents a histopathologic finding that must be considered during the testing and development of new hemoglobin therapeutics, and confirmation of the basic mechanism of lesion development would be helpful in estimating the potential clinical relevance of this finding.

EDITOR'S SUMMARY

Early in the development of DCLHb by Baxter, microscopic subendocardial lesions were seen in necropsy specimens in certain primates (rhesus and cynomolgus monkeys) and pigs. The most sensitive tissue was the left ventricular papillary muscle, followed by the left ventricular free wall and inter-ventricular septum. The right ventricle was sometimes affected, but to a much lesser degree. The atria were almost never affected.

Initially this was though to be a non-specific lesion, similar to one commonly seen in hearts of animals treated with vasoconstrictors such as norepinephrine. Although concern was raised, it was not of sufficient concern to either Baxter or the FDA to curtail clinical testing. Eventually, trauma patients treated with the clinical version of DCLHb (HemAssist™) demonstrated higher mortality than controls, although no direct connection between the heart lesions and any adverse events in humans was found.

A great deal of effort was expended at Baxter to discover the cause of the heart lesions. Many hemoglobin derivatives were studied, but no clear explanation emerged. Finally, Baxter workers were able to reproduce the lesions with the NO synthase inhibitor, L-NAME, and they showed that a new recombinant mutant with reduced NO-binding kinetics also prevented the lesions.

Baxter's conclusion from this work is that the lesion stems from NO scavenging. While the mechanism is still not clearly understood, it is gratifying that the lesions have not been reported so far in any humans who have come to autopsy.

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SECTION

5

Perfluorocarbon-Based Oxygen Carriers

The only blood substitute that has been approved for clinical use was a perfluorocarbon-based product (Fluosol®). Since its efficacy was marginal, it was not successful and it was eventually withdrawn from the market. However the experience was encouraging, and new products followed with greater oxygen capacity. This section reviews the basic chemistry of the perfluorocarbons, and then discusses the products that have been developed to follow Fluosol.

Chapter 24

Fluorocarbon Emulsions as *in vivo* Oxygen Delivery Systems: Background and Chemistry

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FLUOROCARBON CHEMISTRY

The first reported perfluorocarbon (or fluorocarbon, PFC), CF_4, was isolated by Lebeau and Damien (1926). The first kg-sized batches of higher molecular weight (MW) fluorocarbons were produced shortly before World War II (Simons, 1945). Their initial industrial development was for the purpose of handling the extremely corrosive uranium hexafluorides. Their high gas-dissolving capacity was investigated by Hildebrand and coworkers in the late 1940s (Gjaldbaek and Hildebrand, 1949).

Initial applications of PFCs in the biomedical field included uses in cellular microsurgery (Kopac, 1955), blood oxygenation (Howlett *et al.*, 1965), or to isolate viruses (Gessler *et al.*, 1956). Clark and Gollan (1966) then showed that a mouse could survive while breathing an oxygen-saturated liquid PFC, demonstrating that the PFC supported respiration and caused the animal no harm. Because PFCs are virtually insoluble in water, parenteral administration is achieved in the form of PFC-in-water emulsions. The first emulsions and 'blood substitution' experiments were performed by Sloviter and Kamimoto (1967) and Geyer *et al.* (1968). Survival of rats exchange-transfused with a PFC emulsion in a 50% carbon monoxide atmosphere dramatically illustrated the possibility of life without any functional hemoglobin.

Commercial development of injectable PFC emulsion for *in vivo* oxygen delivery followed soon after (perhaps too soon), with the inception of *Fluosol*-DA in the 1970s. More advanced formulations were developed in the early 1990s.

This short review focuses primarily on the chemistry of PFCs and PFC emulsions destined for *in vivo* oxygen delivery. For the sake of space, only the most recent references are generally provided here. For a broader assessment of the context, detailed treatment of the topic and comparison with other approaches to oxygen delivery, the reader is referred to more comprehensive, recently published reviews (Riess, 2001, 2004; Krafft *et al.*, 2003).

Unique chemicals

Fluorocarbons are formally derived from hydrocarbons by replacing all the hydrogen atoms by fluorine atoms. A loose but common definition encompasses highly fluorinated molecules also containing occasional hydrogen, oxygen or nitrogen atoms and halogens other than fluorine. PFCs have unique attributes and performances that derive directly, can be understood and can generally be predicted from the specific electronic

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structure and spatial requirement of the constituent atoms, especially the fluorine atom (Riess, 2002, Riess, 2005).

Replacing all the hydrogen atoms with fluorines in an organic molecule brings about some radical changes in behavior. Fluorine has nine electrons (and nine protons and ten neutrons) as compared to only one electron (and one proton) for hydrogen. These nine electrons are packed in a more compact way, forming a much denser electron cloud. Fluorine has a higher ionization potential than hydrogen (just after the inert gases helium and neon), a considerably larger electron affinity, the highest electronegativity of all atoms, and a lower polarizability than hydrogen – second only to neon.

As a result, perfluoroalkyl chains (*F*-chains) are structurally quite different from conventional alkyl chains. The fluorine atom, being more space demanding than hydrogen, forces the C—C skeleton to adopt a helical arrangement rather than the usual planar zigzag configuration found in hydrocarbon chains (*H*-chains). *F*-chains are also bulkier than *H*-chains (cross sections of *ca*. 30 vs 20 $Å^2$, respectively). The larger trans/gauche interchange energy barrier (4.6 vs 2.0 kJ/mol, respectively) makes them more rigid (it takes more energy to twist them) and allows for fewer kinks.

Stable and inert

The thermal stability and chemical inertness of PFCs reflect a combination of the strength of the C—F bond, low polarizability and strong electro-attracting character of fluorine (which reinforces the C—C backbone), and of the compact, repellent electron shield provided by the fluorine atoms.

PFCs are among the most stable (in the thermodynamic sense) and chemically inert (in the kinetic sense) chemicals known to man. The C—F bond is the strongest single bond found in molecular compounds (e.g. 530 kJ/mol for C—F in C_2F_6 vs 418 kJ/mol for C—H in C_2H_6). Moreover, the extreme electron-attracting character of fluorine enhances the C—C bond energy in the skeleton (e.g. 413 kJ/mol in $CF_3—CF_3$ vs 371 kJ/mol in $CH_3—CH_3$).

From the reactivity standpoint, there are no low energy molecular orbitals accessible for binding O_2, CO or NO. Also, the larger, electronically more dense fluorine atoms cover and protect the C—C backbone and repel approaching reagent much more effectively than hydrogen atoms do (the 'Scotchguard' effect).

The reactivity of PFCs differs widely from that of hydrocarbons (HC). While HCs (think of octane) are highly flammable, PFCs (e.g. *F*-octane) are not. *n*-$C_8F_{17}Br$ (perflubron) withstands 300°C for 24 hours, cooking with 5N H_2SO_4 at 105°C for 10 days, prolonged exposure to 300-nm UV, etc. PFCs are not subject to oxidation, and do not undergo any reaction under the conditions of processing, storage and use relevant to therapeutic oxygen delivery. Poly(tetrafluoroethylene) (PTFE, e.g. Teflon®) is one of the most inert organic materials known. Expanded PTFE (e.g. Gore-Tex®) is used in body implant devices and allows natural tissues to grow in its pores. Fluorinated surfactants can resist highly aggressive media, including strong acids, alkalis and oxidants, even at high temperatures.

Finally, PFCs are not metabolized, and no microorganism is known to feed on them – since Mother Nature did not exploit the PFC route, she did not develop the enzymes that would have been needed to recycle them. Pure PFCs have no effect on cell cultures either, other than the benefits that result from O_2 or CO_2 delivery. One can drink PFCs by the liter without side effects other than wet pants due to extreme spreadability!

F-chains and *H*-chains in surfactants behave differently at interfaces. Thus, the contribution to the free energy of adsorption of a CF_2 segment from water to the air/water interface is roughly twice as large as that of a CH_2 (−5.1 kJ per CF_2 versus −2.6 per CH_2). This reflects the higher interfacial activity of CF_2s (lower surface tension) and their higher affinity for (and alikeness to) gases as compared to CH_2s. The surface tensions of PFCs are significantly lower than those of HCs. Thus the surface tension of *F*-*n*-octane at 25°C is 13.6 mN/m versus 21.1 mN/m for *n*-octane (72 mN/m for water).

Hydrophobic and lipophobic

PFCs are the most hydrophobic organic substances ever invented. They are considerably more hydrophobic than HC oils. Increased hydrophobicity is primarily a matter of low polarizability and, for a given PFC molecular structure, of increased surface area exposed to the surrounding medium, as compared to the

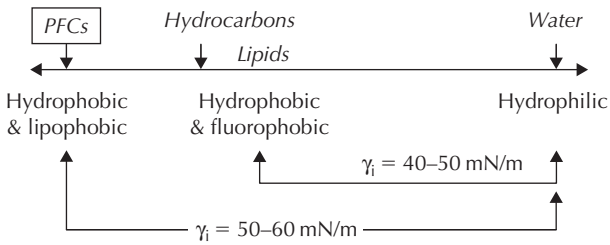


Figure 24.1 The relative positions of fluorocarbons, hydrocarbons and water on a polarity scale: fluorocarbons are more hydrophobic than olive oil, and are oleophobic as well.

'parent' HC. On a polarity scale (where water would be on the high polarity side, Figure 24.1), PFCs are located further out than HCs with respect to water. The PFC/water interfacial tension (which opposes the dispersion/emulsification of PFCs in water) can reach 60 mN/m. The mixing of PFCs and HCs is also highly non-ideal, resulting for PFCs in the unique attribute of being not only extremely hydrophobic but also lipophobic (or oleophobic).

The well-known 'hydrophobic effect' is the basis for the self-assembly of lipids into bilayer membranes, such as cell membranes. Being both strongly hydrophobic and substantially lipophobic, PFCs and *F*-chains tend to keep to themselves and do not tend to mix with either aqueous phases or lipids, which certainly contributes to their biological inertness. For example, *F*-octyl bromide has remarkably low membrane solubility and produces no significant changes in the phospholipid organization (Ellena *et al.*, 2002).

The extreme hydrophobicity of PFCs translates into very low water solubility (typically, one order of magnitude lower than HCs). These low water solubilities, combined with high volatility (that also reflects the low intermolecular forces), are the basis for the stabilization of injectable dispersions of micron-size PFC-containing gas bubbles that serve as *in vivo* reflectors for contrast ultrasound imaging (Riess, 2003; Schutt *et al.*, 2003). Contrary to the water-soluble gases O_2, N_2 and CO_2, the very poorly water-soluble PFC stays in the bubbles and counterbalances blood pressure and pressure due to surface tension, thus preventing rapid microbubble dissolution in the blood. Several such PFC-based contrast agents have been licensed by the FDA in the United States or EMEA in Europe, and are now commercially available.

OXYGEN DISSOLVING AND DELIVERING CAPACITY: GAS-LIKE LIQUIDS

PFCs dissolve gases better than any other liquid. There exists no possibility for PFCs to *bind* gases chemically. The exceptionally large gas-dissolving capacity of PFCs derives from fluorine's extremely low polarizability, which translates into low van der Waals interactions between PFC molecules, as van der Waals interactions depend directly on fluctuations in polarity of the electronic cloud. Since van der Waals interactions are the only *inter*molecular forces that keep together nonpolar molecules, the intermolecular forces in PFCs are very feeble – in sharp contrast with their strong *intra*molecular bonds. Consequently, liquid PFCs behave like gas-like fluids. They easily dissolve other substances of similarly low cohesivity, namely gases, including O_2, CO_2, N_2, NO, etc. The low cohesivity of PFCs is also reflected by low boiling points and high volatility relative to their molecular weight. Their Hildebrand parameters (which express the cohesive energy density of fluids, hence their aptitude for mutual solubility) of oxygen (5.7), typical PFCs (~6) and HCs (7–9), and water (23.5) indicate that PFCs are very much like O_2, more so than HCs, not to speak of water, which has a highly structured three-dimensional network of strong hydrogen bonds. In other words, it takes less energy to create a hole in a less cohesive material, such as a PFC, and host a guest of similar cohesiveness, such as O_2.

On an O_2 solubility scale (Figure 24.2), water is at one end of the scale while the gas-like PFCs are at the other end. HCs lie in-between. There is roughly a factor 20 in terms of volume% O_2 solubility between PFCs and water, and a factor 2 between PFCs and HCs. Oxygen solubility decreases with increasing molecular weight of the PFC. Linear PFCs have a larger O_2 solubility than cyclic PFCs of comparable molecular weight. The O_2 solubilities of the PFCs pertinent to intravascular use range from 40 to 50 volume per cent under 1 atmosphere. Recent O_2 solubility measurements can be found in Costa-Gomez *et al.* (2004). The rate of uptake and release of O_2 by PFCs is essentially insensitive to temperature and environment. The CO_2 solubilities of PFCs range from 140 to 240 volume per cent.

Figure 24.3 reminds us that gas dissolution in PFCs follows Henry's law (i.e., dissolved O_2 concentration at equilibrium – or solubility – at a given temperature is directly proportional to the

gas's partial pressure) and does not depend on a localized chemical bond as in hemoglobin. Consequently, O_2 can be rapidly and extensively extracted from PFCs when needed. Figure 24.3 also reminds us that high inspired O_2 fractions should be used in order to maximize the benefits of PFC emulsion administration to the patient.

Superior O_2 solubilities would not suffice to make PFCs good candidate *in vivo* O_2 carriers; it is the *combination* of O_2/CO_2 solubilities *and* of exceptional biological inertness that creates the potential.

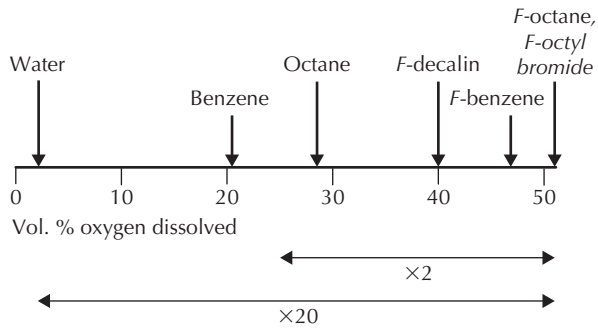


Figure 24.2 An oxygen solubility scale. Fluorocarbons, because they have low cohesiveness, dissolve oxygen most effectively. Water, a highly cohesive liquid due to extensive hydrogen bonding, is an exceptionally poor oxygen solvent.

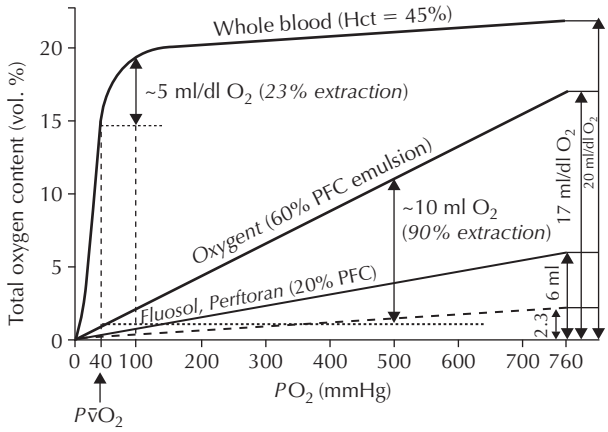


Figure 24.3 Oxygen solubility in fluorocarbons follows Henry's law – i.e., it is directly proportional to the gas' partial pressure, as expected in the absence of chemical bonding, while oxygen uptake by hemoglobin, which binds oxygen through a strong covalent bond to its iron atoms, follows a sigmoid curve that saturates when the partial pressure of oxygen in earth's atmosphere is attained. Oxygen extraction from a PFC emulsion can reach 90 per cent of oxygen content.

In summary, while the properties and behavior of PFCs (and of perfluoroalkylated compounds) are, in essence, of the same nature as those of regular organic compounds, the exceptionally strong intramolecular binding and uniquely low intermolecular cohesiveness of liquid PFCs result in unique properties. Many *F*-compounds can reach a level of effectiveness in their performance that leads to technological feats that cannot be achieved with non-fluorinated materials. Compared to HCs, PFCs are typically *much* more inert, have higher densities, compressibilities, fluidity, spreading coefficients and gas-dissolving capacities, and lower refraction indexes, surface tensions, dielectric constants and water solubilities. Moreover, *F*-compounds offer unique *combinations* of properties that can make them irreplaceable and constitute the basis for further potential biomedical applications (Riess, 2002, 2004).

PERFLUOROCARBON EMULSIONS FOR *IN VIVO* OXYGEN TRANSPORT

The principal challenges in the development of injectable PFC-in-water emulsions include selecting a PFC that is readily excretable, easy to manufacture in a highly pure state, and easy to emulsify; preparing stable, small-sized heat-sterilizable emulsions using a surfactant that, preferably, is well accepted in the pharmaceutical industry; controlling emulsion droplet size, and counteracting molecular diffusion, which is responsible for particle size growth over time. It is important that the *in vivo* behavior of the emulsion is understood and its side effects minimized, and conditions of use for clinical evaluation and optimal benefit to the patient must be defined. Also significant are cost effectiveness, user friendliness, and compliance with current Good Manufacturing Practices and regulations from health authorities (Krafft *et al.*, 1998; Riess, 2001).

Optimal perfluorocarbons for clinical emulsions

There are, *a priori*, many PFCs to choose from since, in principle, almost any molecular structure can be synthesized. Actually, very few PFCs are acceptable for parenteral use – PFCs that optimally combine rapid excretion with the capability of producing stable emulsions. It has been determined that rapid excretion of the PFC requires a touch of lipid solubility, meaning that the PFC

should not be too heavy in terms of molecular weight. On the other hand, emulsion stability requires low water solubility, hence the PFC must not be too light. These two conditions – relatively high lipid solubility and the lowest possible water solubility – are clearly difficult to satisfy simultaneously. Vapor pressure, which also depends on molecular weight, is an important parameter; too light a PFC can favor retention of air in the alveoli, resulting in increased pulmonary residual volume (also known as pulmonary gas trapping). In order to avoid this phenomenon, the vapor pressure of the PFC phase at body temperature should probably not exceed about 10 mmHg.

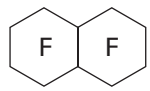
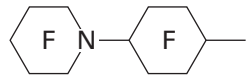
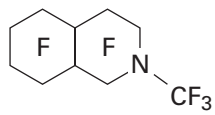
Table 24.1 Characteristics of fluorocarbon emulsions related to the dispersed fluorocarbon(s)

- Synthetic sterile raw material
- Dissolved oxygen – Readily and immediately available
  – High extraction ratio
- Linear O_2 versus PO_2 uptake – No saturation
- Passive delivery, no binding of CO, NO
- CO_2 dissolution and removal
- No metabolism – Respiratory excretion of fluorocarbon
- Emulsion stability increases with increasing molecular weight of fluorocarbon
- Fluorocarbon excretion rate increases with decreasing molecular weight and increasing lipophilicity of fluorocarbon

Table 24.1 summarizes the key features of PFC emulsions that depend on those of the dispersed PFC. Table 24.2 displays some characteristics of the PFCs that have been most extensively investigated as candidate O_2 carriers.

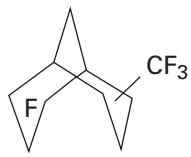
Table 24.3 collects PFC emulsions that have reached some degree of commercial development. Figure 24.4 reminds us that the organ retention of PFCs is primarily an exponential function of molecular weight, with cyclization, branching and the presence of heteroatoms within their structure having little effect on excretion rate other than through their effect on molecular weight.

Table 24.2 Fluorocarbons most thoroughly investigated for injectable oxygen carriers development

	Structural formula[a]	Code name (MW)	Preparation procedure (Purity)	Boiling point (°C) Vapor pressure, (torr, 37°C)	Solubility O_2 CO_2 (vol%, 37°C)	Excretion half-time (days)[b]
1	$CF_3(CF_2)_7Br$	PFOB Perflubron (499)	Telomerization (>99%)	143 11	50 210	~4
2	$CF_3(CF_2)_9Br$	PFDB (599)	Telomerization (>98%)	180 1.5		20–25
3	$Cl(CF_2)_8Cl$	PFDCO (471)	Undisclosed	155 6	43 –	~7
4		FDC (462)	CoF_3 (97%, cis + trans)	142 12.5	42 142	~7
5	$N[(CF_2)_2CF_3]_3$	FTPA (521)	Electrochem. (>95%)	131 18	45 166	~65
6	$N[(CF_2)_3CF_3]_3$	FTBA (671)	Electrochem. (80–85%)	178 1.1	38 140	>500
7		FMCP (596)	Electrochem. (~55%)	168 2	40	~90
8		FMIQ (495)	Electrochem. (~95%)	153–154	42	~11

(*Continued*)

Table 24.2 *Continued*

	Structural formula[a]	Code name (MW)	Preparation procedure (Purity)	Boiling point (°C) Vapor pressure 37°C)	Solubility O_2 CO_2 (vol%, 37°C)	Excretion half-time (days)[b]
9		FDN (512)	CoF_3 (50–55%)	157 6	40	~14
10	$CF_3(CF_2)_3CH{=}CH(CF_2)_3CF_3$	F-44E (464)	Telomerization (>99%)	−12.5	50 247	~7
11	C_5F_{12}	(288)	Direct fluorination	29	[c]	<2 hours

[a] The symbol F within a cycle indicates that all carbons in the cycle are perfluorinated. [b] Depends significantly on dose; see original papers. [c] Used for gas bubble stabilization.

Table 24.3 Perfluorocarbon emulsions having reached some degree of commercial development

Trade name	Company	Perfluorocarbon[a]	Concentration v/v (w/v)	Surfactants	Remarks	Status (July 2004)
Fluosol®	Green Cross Corp. (Japan)	FDC/FTPA 7:3 ***4/5***	11% (20%)	Pluronic F68 EYP[b] K oleate	Frozen stem emulsion Reconstitute	Approved in the US for PTCA 1989 Discontinued
Oxypherol® (Fluosol-43)	Green Cross Corp. (Japan)	FTBA ***6***	11% (20%)	Pluronic F68	High organ retention	For experimental use; discontinued
Perftoran	Perftoran Co. (Russia)	FDC/FMCP 7:3 ***4/7***	11% (20%)	Poloxamer EYP	High organ retention of ***7;*** frozen storage	Approved in Russia 1996
FMIQ emulsion	Green Cross Corp. (Japan)	FMIQ ***8***	13% (25%)	EYP K oleate		Not developed
Addox®	Adamantech (USA)	'FMA'[c] FDN ***9***	21% (40%)	EYP	Low PFC definition	Abandoned
Therox®	DuPont (USA)	F-44E ***10***	40% (78%)	EYP	For research only	Discontinued
Oxygent™ (AF0144)	Alliance Pharmaceutical Corp. (USA)	PFOB/PFDB ***1/2***	32% (60%)	EYP	Stabilized with PFDB	Completed a Phase III in general surgery
Oxyfluor®	HemaGen (USA)	PFDCO ***3***	40% (78%)	EYP	Stabilized with saffoil	Phase II clinical trials in CPB Abandoned
Oxycyte	Synthetic Blood Intl (USA)	Cyclic $C_{10}F_{20}$	33% (60%)	EYP		Phase I study

[a] See Table 24.2 for abbreviations. [b] EYP = egg yolk phospholipids. [c] 'FMA' perfluoromethyladamantane.

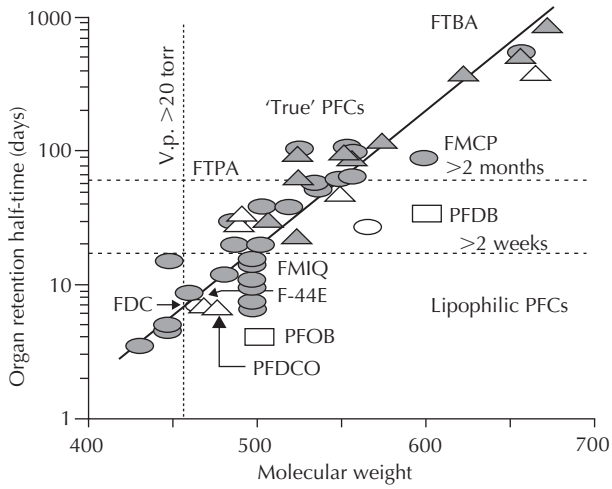


Figure 24.4 Organ retention of true fluorocarbons is essentially an exponential function of molecular weight. Fluorocarbons containing lipophilic elements (squares), such as a terminal bromine atom or two chlorine atoms, are excreted more rapidly than would be predicted on the sole basis of their molecular weight.

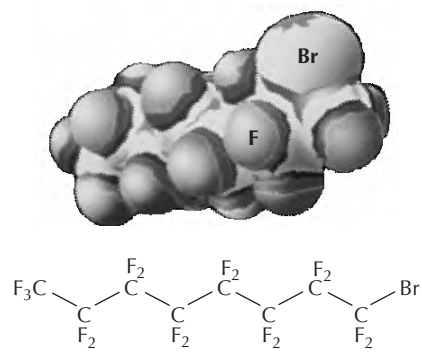


Figure 24.5 Molecular model of *F*-octyl bromide, showing the lipophilic bromine atom protruding at the end of the linear chain.

There are, however, a few valuable exceptions to this rule, i.e., PFCs that are excreted more rapidly than would be predicted on the sole basis of their molecular weight. This is the case of *F*-octyl bromide (PFOB, perflubron). *F*-octyl bromide is slightly more lipophilic than a standard PFC of the same molecular weight, due to its well-exposed, polarizable terminal bromine atom (Figure 24.5), which facilitates carriage of the molecule by circulating lipoproteins. This lesser lipophobicity of *F*-octyl bromide, as compared to *F*-decalin, is, for example, reflected by an eight times larger solubility in olive oil and by a *ca*. 40°C lower critical solution temperature in hexane (Table 24.4). Table 24.4 clearly shows that all PFCs are not equivalent.

Table 24.4 Comparison of some physical properties of *F*-octyl bromide and *F*-decalin

Property (units)	Symbol	PFOB	FDC (cis + trans)
Molecular formula		$C_8F_{17}Br$	$C_{10}F_{18}$
Molecular weight (g/mol)	M_w	499	462
Melting point (°C)	*m.p.*	5	−10
Vapor pressure (torr, 37°C)	*v.p.*	10.5	14
Kinematic viscosity (centistokes, 25°C)	V	1.0	2.9
Interfacial tension versus saline (mN/m)	γ_i	51.3	~60
Spreading coefficient (mN/m)	S (o/w)	+2.7	−1.5
O_2 solubility (vol.%, 25°C)	$[O_2]$	50	40
CO_2 solubility (vol.%, 25°C)	$[CO_2]$	~210	~140
Critical solution temperature (*n*-hexane, °C)	CST (hexane)	−20	+22
Solubility in water (mmol/l)		5.10^{-6}	10.10^{-6}
Solubility in olive oil (mmol/l)		37	4.6

At this point, PFOB appears to offer the best combination (among the PFCs investigated) of rapid excretion, ability to form stable emulsions with phospholipids, and easy high-purity manufacture. Additionally, it has among the largest O_2 and CO_2 solubilities relative to its molecular weight.

Synthesis of PFCS

Reliable access to raw material, ease of manufacture, and cost-effectiveness will be critical in determining the degree of acceptance, breadth of application and commercial success of an O_2 carrier. PFCs have the advantage of being totally synthetic materials, and hence do not depend on the collection of human or animal blood. There exist well-established synthetic routes that allow production of PFCs within tight specifications in high tonnages and in very high purity. Industrial access to PFOB ***1*** (see Table 24.2 for the code numbers assigned to the various PFCs) is achieved

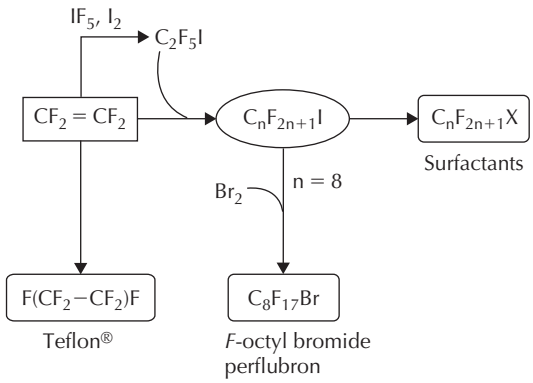


Figure 24.6 Industrial process for *F*-octyl bromide (perflubron) synthesis: one step from a pivotal perfluorochemical, *F*-octyl iodide.

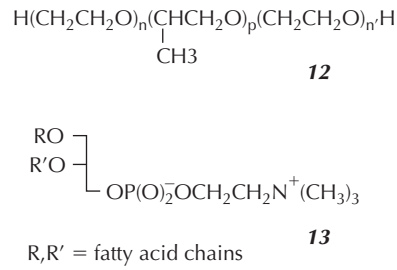


Figure 24.7 The only two surfactants used in PFC emulsion development, poloxamers and phospholipids.

via simple bromination of a key intermediate, *F*-octyl iodide, on the route to large-tonnage industrial fluorosurfactants (Figure 24.6). The telomerization process used for manufacturing *F*-octyl iodide from tetrafluoroethylene, CF_2CF_2 (the monomer of Teflon), ensures that perflubron can be produced on a multi-ton scale in better than 99.9 per cent purity.

Optimal emulsifiers

Proper selection of an effective, biocompatible emulsifier or emulsifier system is essential for successful PFC emulsion development. The emulsifier reduces the interfacial tension, γ_i, that opposes the dispersion of the very hydrophobic PFC. It also stabilizes the emulsion once it is formed. The only two surfactants used in PFC emulsion development so far are poloxamers and phospholipids (Figure 24.7).

Poloxamers ***12*** are neutral block copolymers. Poloxamer 188 (e.g. Pluronic F68®) was used in the early PFC emulsions (e.g. Fluosol), but its surface activity is relatively poor, translating into low emulsion stability; the purity of the commercial products is usually rather low; its cloud point (~110–115°C) prevents sterilization at 121°C; its tendency to form gels limits the PFC concentration in the emulsions; and finally, Pluronic F68 has been found responsible for the transient complement activation-mediated reaction observed in some patients in response to the injection of Fluosol.

Egg-yolk phospholipids (EYP), whose major components are the amphoteric phosphatidylcholines ***13***, provide significantly better emulsion stability than poloxamers. The stabilization effect is particularly remarkable with PFOB. No complement activation was reported (Noveck *et al.*, 2000). Phospholipids have a long history of use in pharmaceuticals; their pharmacology is well documented and there exist reliable commercial sources of pharmaceutical grade EYP. The hydrolysis of EYP in PFC emulsions is minimal when pH is close to neutral; their oxidation is usually minimized by addition of an antioxidant (e.g. α-d-tocopherol), and through manufacture and packaging under nitrogen.

Production of small, stable emulsion particles

Injectable PFC emulsions need to be stable, small sized, narrowly dispersed, sterile, and easy to manufacture consistently. The presently developed PFC emulsions share several key features with the fat emulsions (e.g. Intralipid®) commonly used for parenteral nutrition, including use of the same emulsifier, EYP, and the same production process through high-shear homogenization, followed by terminal heat sterilization (Figure 24.8).

Emulsifying PFCs implies counteracting the large interfacial tension, γ_i, of 50–60 mN/m that stands against dispersion of PFCs in water. The target PFC droplets are in the 0.1–0.2 μm range and are typically covered with a monomolecular film of the phospholipids. User-friendly commercial PFC emulsions need to be ready for use and to remain stable for at least 2 years without significant changes in particle sizes and particle size distribution. Frozen storage, thawing and reconstitution (as was the case for Fluosol) are clearly impractical and unacceptable. Technology for large-scale production of injectable emulsions in compliance with good manufacturing practices is well established in the pharmaceutical industry.

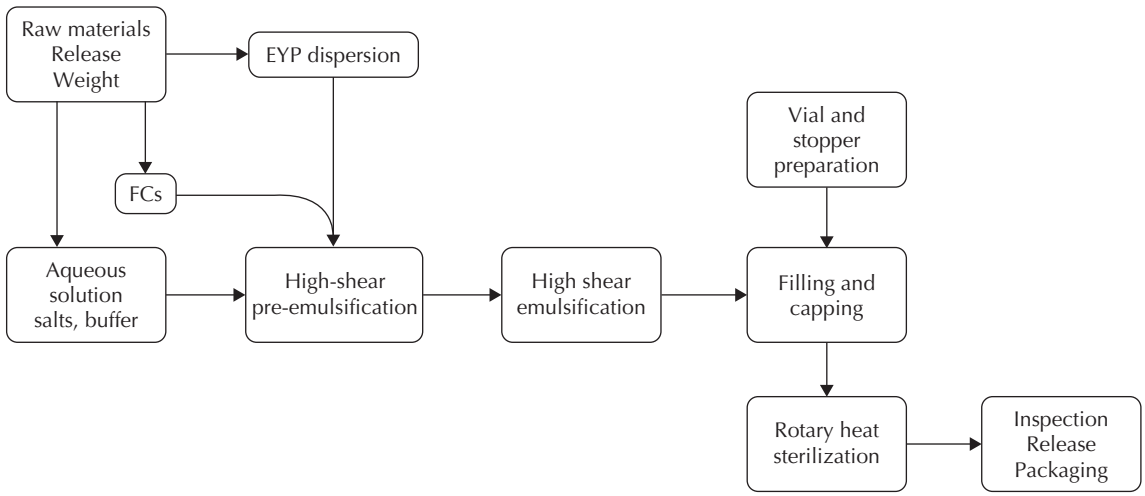


Figure 24.8 Schematic fluorocarbon emulsion production flowchart.

For EYP-based PFC emulsions, the first step of the process involves dispersing the water-insoluble phospholipids in a saline solution (Figure 24.8). The PFC is then added to this saline phase, where it is broken down into fairly large droplets (average ~5 μm) with a high-shear rotor-stator type homogenizer. This premix undergoes final emulsification using a high-pressure homogenizer that provides a high energy density. Minimal exposure to oxygen (through nitrogen sparging and blanketing), pyrogen-free water-for-injection and a particulate-free environment are used throughout processing. The Gaulin-type high-pressure homogenizer is easy to control, gives narrow, consistent particle size distributions, and can be operated on very large scales. The formulation process for PFC emulsions being additive, its yield is essentially quantitative with respect to the raw materials utilized. The present products are terminally heat-sterilized in standard conditions. Manufacture of a practical, small-sized, narrowly dispersed, sterile PFC emulsion for parenteral use, although it relies on existing technologies, nevertheless requires the development of specific know-how.

Stability: counteracting molecular diffusion and coalescence

Over time, submicronic PFC droplets usually grow as a result of molecular diffusion (also known as Ostwald ripening or isothermal distillation; Figure 24.9a) rather than through droplet coalescence (Figure 24.9b). In this process, individual PFC molecules leave the smaller droplets, where their chemical potential is higher due to higher curvature, to join larger droplets, where curvature is smaller.

Droplet growth by molecular diffusion follows the Lifshitz–Slezov equation:

$$\frac{d\bar{r}^3}{dt} = \omega = \frac{8VmCD\gamma_i}{9RT} f(\varphi)$$

which says that the average droplet volume $\bar{r}^3$ in a given emulsion increases over time t proportionally to the water/PFC interfacial tension γ_i and to the solubility C and diffusibility D of the PFC in the aqueous phase (Vm is the mole volume of the PFC and $f(\varphi)$ introduces the effect of its volume fraction). Molecular diffusion is characterized by a linear increase of $\bar{r}^3$ over time, and by a time-invariant droplet size distribution function.

By chance, phospholipids are particularly apt at reducing γ_i. Additionally, the solubility C of the PFC phase in water can be reduced by adding a 'heavier' (higher molecular weight PFC (Figure 24.10). The longer organ retention of this higher molecular weight PFC can be mitigated by using a somewhat lipophilic PFC (see below). Droplet coalescence may contribute to instability of some emulsions when mechanical stress is applied and at higher temperatures, as during heat sterilization. Sedimentation and flocculation are fully reversible and usually pose no problem. Table 24.5 summarizes the properties of PFC emulsions that are related to their being a dispersion of droplets.

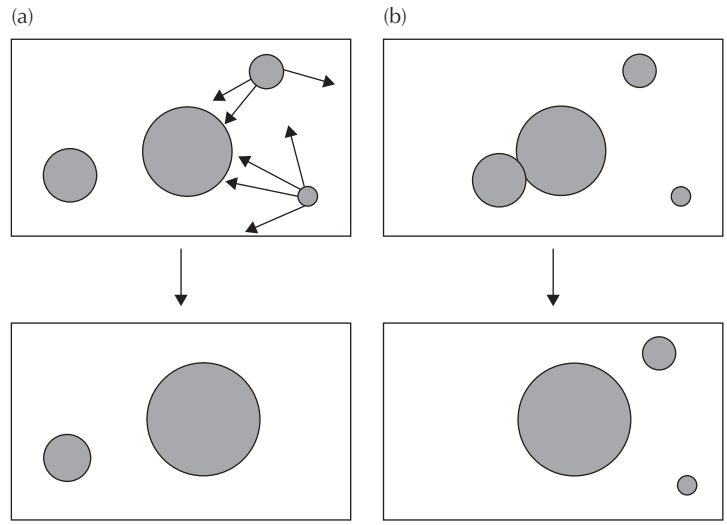


Figure 24.9 Particle size growth over time in fluorocarbon emulsions is due to molecular diffusion (a) rather than to droplet coalescence (b). The thin arrows in scheme (a) represent individual molecules leaving the smaller droplets to join larger ones, where the chemical potential is lower.

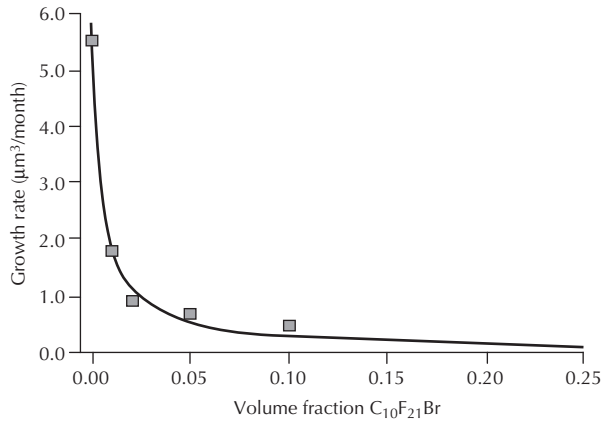


Figure 24.10 Droplet growth in an *F*-octyl bromide emulsion can be effectively repressed by adding a small amount of *F*-decyl bromide (from Weers *et al.*, 1994).

Table 24.5 Fluorocarbon emulsion characteristics related to their particulate nature

- Small sizes/RBC (0.15–0.2 μm versus 7 μm), yet no extravasation
- Numerous particles – facilitate oxygen diffusion
- Adjustable viscosity, close to that of blood
- Mechanical resistance (pumps, filters)
- Foreign particles – RES clearance
  - Can activate macrophages (flu-like symptoms)
  - Short intravascular persistence
- Straightforward large-scale manufacturing
- Terminal heat sterilization

DEVELOPMENT OF INJECTABLE FLUOROCARBON EMULSIONS

Fluosol-DA related emulsions

The first commercial development efforts (Fluosol-DA® in Japan, Perftoran® in the Soviet Union, Emulsion No. II in China) involved use of *F*-decalin ***4*** as the primary PFC and of a poloxamer (e.g. Pluronic F68) as the primary emulsifier. The main limitations of these emulsions included the presence of large amounts, typically 30 per cent, of a heavy PFC (***5*** or ***7***) that reduces molecular diffusion but has an organ retention half-life of several months, excessive dilution, and poor stability. Pluronic F68 caused complement activation-type side effects. Fluosol (Table 24.6) came as a frozen emulsion concentrate that needed to be thawed and mixed with two annex solutions prior to administration; it then had to be used within a few hours. These constraints certainly had a part in Fluosol's commercial failure. Oxypherol, a 25 per cent w/v emulsion of *F*-tributylamine ***6***, was highly stable, but not intended for human use due to excessively long organ retention. A ready-for-use EYP-based emulsion of *F-N*-methylisoquinoline ***8*** was investigated, but not developed commercially. Perftoran has been licensed for use in Russia (Ivanitsky, 2001; see Chapter 26). Recent experimental work with Perftoran includes microvascular gas embolism clearance studies following emulsion administration (Eckman and Lomivorotov, 2003).

Table 24.6 Emulsion formulations (w/v) – Fluosol® and Oxygent™

Function	Components	Fluosol	Oxygent
Oxygen carriers	*F*-Decalin ***4***	14.0[a]	
	+ *F*-Tripropylamine ***5***	6.0[a]	
	F-Octyl bromide ***1*** + *F*-Decyl bromide ***2***	}	60
Emulsifiers	Pluronic F-68	2.7[a]	
	Egg yolk phospholipids	0.4[a]	3.6
	Potassium oleate	0.03[a]	
Cryoprotector	Glycerol	0.8[b]	
Ionic balance, pH and osmotic pressure control	NaCl	0.60[b]	0.25
	KCl	0.034[c]	
	$MgCl_2$	0.020[b]	
	$CaCl_2$	0.028[b]	
	$NaHCO_3$	0.21[c]	[d]
	Dextrose	0.18[b]	

[a] From stem emulsion; [b] From annex solution H; [c] From annex solution C; [d] Phosphate buffer.

Oxygent™

Oxygent™ AF0144 (Alliance Pharmaceutical Corp., San Diego, CA; see Table 24.6) has essentially overcome the above limitations. It is a 60 per cent w/v concentrated emulsion, primarily of PFOB ***1***. It has an average droplet size of 0.16 μm after heat sterilization, and a viscosity around 5 cP (extrapolated at zero shear rate) – i.e., somewhat above that of water. Its pH and osmolarity are adjusted to 7.1 and 304 mOsm, respectively. Adequate shelf stability (over 2 years at 5–10°C), without excessive organ retention, is achieved by adding a few per cent of the slightly lipophilic heavier *F*-decyl bromide ***2***, a higher homologue of ***1*** (Figure 24.10).

The emulsion is terminally steam-sterilized in a rotary autoclave above 121°C, using a procedure that achieves uniform heat penetration, maintains emulsion integrity and provides the required probability of less than one non-sterile unit in one million. Oxygent is provided ready for use.

No effect on immune function has been seen in healthy volunteers (Noveck *et al.*, 2000) and no evidence of pulmonary trapping or other pulmonary side effect has been found in man.

A randomized, multicenter, European Phase III clinical evaluation of Oxygent in general surgery patients has established its ability to significantly reduce and avoid red blood cell transfusion. The trial was conducted using an augmented acute normovolemic hemodilution with PFC emulsion protocol. In the protocol-defined target population (330 subjects with blood loss ⩾20 ml/kg body weight), significantly greater avoidance of any red blood cell transfusion, as compared to controls, was maintained through day 21 or day of hospital discharge ($P < 0.05$). There was also a significant reduction in the number of units of blood transfused ($P < 0.001$; Spahn *et al.*, 2002). From the clinical data collected, the hemoglobin equivalency, in terms of added O_2-delivering capacity of a 1-g PFC/kg body weight dose, was estimated to be around 1.5 g hemoglobin (Faithfull, 2003). However, the voluntary suspension of a cardiopulmonary bypass surgery trial with Oxygent, because of side effects assigned to an inadequate clinical protocol that resulted in overly aggressive autologous blood harvesting in the treatment group prior to bypass, was a setback in the development of this emulsion (see Chapter 28). Clinical development is now being resumed by Double-Crane Pharmaceutical, China.

Recent experimental work with Oxygent has demonstrated significant improvement of cerebral oxygenation and mitochondrial function after traumatic brain injury (Daugherty *et al.*, 2004). Resuscitation with Oxygent was superior to stored blood or a plasma expander with respect to restoration of hepatocellular energy metabolism (Paxian *et al.*, 2003). Proper oxygenation of the gastrointestinal system was preserved in cardiac surgical patients administered with the emulsion, demonstrating the potential for Oxygent to prevent perioperative tissue hypoxia (Frumento *et al.*, 2002). Post-dive intravenous treatment with the emulsion decreased the incidence of decompression sickness (Dromsky *et al.*, 2004). The oxygenated emulsion reduced sickle red blood cell-induced vaso-occlusion in an *ex vivo* animal model (Kaul *et al.*, 2001).

Oxyfluor

The developers of Oxyfluor (HemaGen, St. Louis, MO) initially selected *F*-decalin as the O_2 carrier, but soon switched to *F*-α,ω-dichlorooctane ***3*** (40 per cent v/v), the lipophilicity and excretion rate of which are comparable to those of *F*-octyl bromide ***1***. The surfactant was EYP. The particularity of this emulsion was the presence of a small percentage of triglycerides (saffoil) in the formulation that was expected to improve

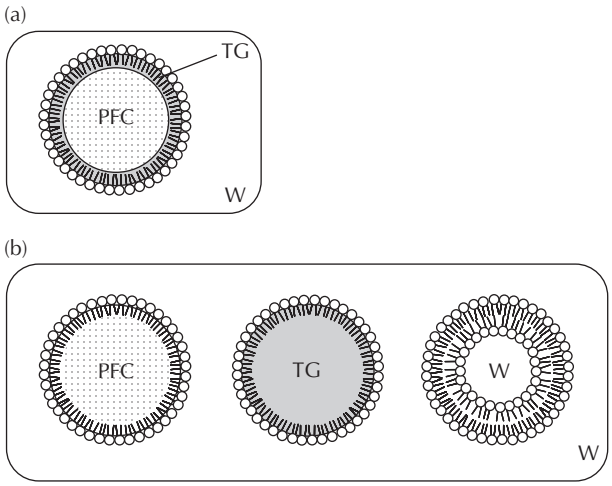


Figure 24.11 Addition of triglycerides to a fluorocarbon/phospholipids emulsion formulation can lead (a) to a 'three-phase' emulsion, or (b) to a mixture of fluorocarbon droplets, hydrocarbon (triglyceride, TG) droplets and empty phospholipid vesicles (adapted from Weers *et al.*, 2004); W = water.

emulsion stability (Kaufman, 1995; Herren *et al.*, 1998).

This emulsion may actually have been based on a misconception: the intent was to achieve a 'three-phase' emulsion in which the added triglycerides would coat the emulsion droplets and improve stability (Figure 24.11a). A recent study has, however, demonstrated that addition of long-chain triglycerides to similarly formulated *F*-decalin or *F*-octyl bromide emulsions actually led to two distinct droplet populations: a PFC emulsion and a lipid emulsion (Figure 24.11b); empty phospholipid vesicles were also present. No stabilization was observed (Weers *et al.*, 2004). The triglyceride droplets, which scatter light much more effectively than the PFC droplets, appear actually to mask the growth of the PFC droplets when emulsion sizing is achieved by quasi-elastic light scattering, leading to gross underestimation of the increase in PFC droplet size over time. Moreover, the addition of the triglycerides led to a substantial increase in emulsion viscosity. Interestingly, a true, single-population 'three-phase' emulsion was obtained when adding medium-sized triglycerides. However, this emulsion was unstable with respect to coalescence, resulting in breakdown during heat sterilization or transportation-mimicking shaking experiments (Weers *et al.*, 2004). The development of Oxyfluor was halted after having reached Phase II clinical trials.

Miscellaneous emulsions

Several other EYP-based PFC emulsions have been reported. Addox®, a 40 per cent w/v emulsion (Adamantech, Inc., Marcus Hook, PA) initially used cyclic products derived from CoF_3 fluorination of dimethyladamantane, but the tissue residence half-life of more than 50 days was deemed unacceptable, and the PFC mixture derived monomethyladamantane fluorination (including ***9***) was subsequently investigated. This emulsion suffered from poor PFC definition, and probably also from poor stability.

Therox®, a concentrated emulsion of bis(*F*-butyl)ethene ***10*** (F-44E) (48 per cent v/v or 83 per cent w/v) having a particle size of 0.25 μm, was developed by DuPont (Wilmington, DE) for research purposes. Another F-44E emulsion has been investigated by Neuron Therapeutics Inc. for treatment of stroke by intrathecal administration of the O_2 carrier.

Oxycyte, an EYP-based formulation of a cyclic $C_{10}F_{20}$ PFC, is in early development by Synthetic Blood Int. (Costa Mesa, CA). Few data are available yet on this emulsion. The molecular weight (500) of the monocyclic, non-lipophilic PFC entails rather prolonged organ retention with no obvious advantage. Oxygen delivery and consumption in the microcirculation was established in the hemodiluted hamster (Cabrales *et al.*, 2004).

THE SEARCH FOR IMPROVED EMULSIONS

Fluorocarbon–hydrocarbon diblock-stabilized emulsions

F-octye bromide still appears to stand out as the best candidate PFC for *in vivo* O_2 delivery. Further stability with respect to Oxygent was gained by supplementing standard phospholipids with mixed fluorocarbon–hydrocarbon diblock compounds, e.g. $C_6F_{13}C_{10}H_{21}$ (F6H10, ***14***; Figure 24.12). An involvement of the diblock molecules at the water/PFC interface, rather than only a slowing down of molecular diffusion due to lowered water solubility of the PFC phase, has been demonstrated. Evidence for such involvement includes a dramatic decrease in PFC/water interfacial tension (typically from about 24 to

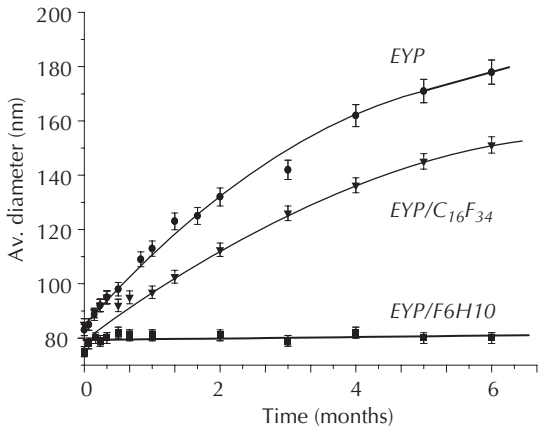


Figure 24.12 Fluorocarbon-hydrocarbon diblock molecules can stabilize fluorocarbon emulsions very effectively. Stabilization with diblock $C_6F_{13}C_{10}H_{21}$ (F6H10) is more effective than with a heavy PFC of similar molecular weight, $C_{16}F_{34}$, which only reduces the solubility of the dispersed PFC phase but not the interfacial tension (from Krafft *et al.*, 2004). Note the preservation of very small particle sizes over time when the diblock is used.

2 mN/m) between PFOB and aqueous phospholipid solutions when a diblock was added to the PFC phase; the observation that the emulsion stabilization effect of a given diblock depends on the length of the lipid's fatty acid chains; and the fact that, when the fit between lipid and diblock alkyl chain length is inadequate, a droplet coalescence mechanism sets in that actually leads to emulsion destabilization. By contrast, the stabilization effect of a heavier PFC that only reduces the solubility of the PFC phase in the aqueous phase was independent of the phospholipid chain length (Marie Bertilla *et al.*, 2004). Because they are substantially lipophilic, FnHm diblocks are excreted rather rapidly. No overt toxicity was seen at doses that are about two orders of magnitude larger then that required for emulsion stabilization (Riess *et al.*, 1994). Improved tissue oxygenation was demonstrated using an F6H10-stabilized *F*-octyl bromide emulsion in a rabbit model of resuscitation from acute hemorrhagic shock (Audonnet-Blaise *et al.*, 2004). No perturbation of the hemodynamic or rheological parameters was induced, even at very large doses. Successful long-term normothermic preservation of the intestine and of organ blocks has been obtained (DeRoover *et al.*, 2001).

Phase-shift emulsions

A so-called 'phase-shift' emulsion of *F*-pentane ***11*** (boiling point 29°C) that turns into gaseous microbubbles at body temperature and was initially intended as a contrast agent for ultrasound imaging is now being investigated for O_2-delivery (Lundgren *et al.*, 2004). The fast permeating gases inside the bubble, i.e., O_2 and CO_2, equilibrate rapidly with the gases dissolved in the plasma and surrounding tissues, allowing O_2 to be carried from the lungs to the tissues. The role of the PFC (a slowly permeating gas) is here no longer to dissolve O_2, but to stabilize O_2 microbubbles osmotically *in vivo* (Figure 24.13; van Liew and Burkard, 1997; Schutt *et al.*, 2003).

Experimental proof of concept includes survival of normovolemic erythrocyte-depleted rats and pigs, and of pigs with potentially lethal hemorrhagic shock and with severe right-to-left shunt (Lundgren *et al.*, 2004). Administration of the *F*-pentane emulsion, along with carbogen breathing, led to suppression of resistance to radiation of hypoxic cells in a rat tumor model (Koch *et al.*, 2002).

However, withdrawal of a new drug application in the United States for use of this product as an ultrasound contrast agent may indicate that safety was not established. The mechanism for prolonged O_2 delivery (at least 2 hours) also remains unclear in view of the short intravascular life of *F*-pentane. Indeed, diagnostic-size doses of *F*-pentane, administered in the form of an 'activated' (gas) emulsion for ultrasound imaging, were determined to have an elimination half-life of only around 2 minutes; *F*-pentane recovery in the expired air was almost complete after 2 hours (Correas *et al.*, 2001). There is little doubt that stabilized O_2 microbubbles can contribute to tissue oxygenation. However, emulsion formulation and stability, and *in vivo* bubble size control, warrant further research.

Further applications of PFC-stabilized microbubbles (gas emulsions) are being investigated for treatment of vascular thrombosis and site-specific drug and gene delivery (Riess, 2004; Unger *et al.*, 2004).

Further research on fluorocarbon emulsions and applications

Droplet coalescence in PFC and HC emulsions stabilized by water-soluble poloxamers or a bis(*F*-alkylated) polyethylene glycol (PEG) has

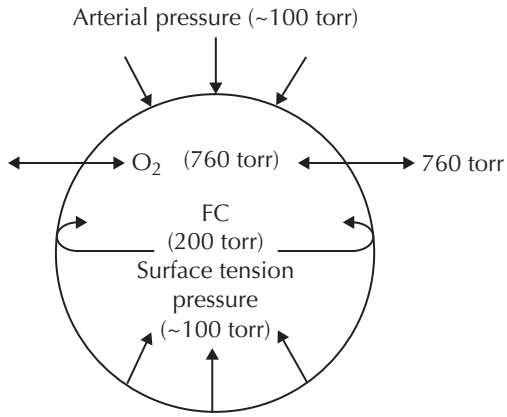


Figure 24.13 Due to extremely low water solubility, volatile fluorocarbons can stabilize injectable oxygen microbubbles against rapid dissolution.

been investigated (Shchukin *et al.*, 2001). Droplet resistance to coalescence was enhanced by a 'deficiency in affinity' between the hydrophobic moiety of the surfactant and the dispersed hydrophobic phase, which pushes the surfactant to organize into a compact, mechanically resistant interfacial structure. An *H*-surfactant was in this respect significantly more effective in stabilizing an PFC emulsion against coalescence than an *F*-surfactant whose *F*-tails tend to dissolve loosely in the PFC. However, these considerations do not appear to apply to the practical PFC emulsions in development, which are formulated with phospholipids that are essentially insoluble in both polar and non-polar phases.

A further research objective is to prolong the intravascular persistence of PFC emulsions. *In vitro* phagocytosis of a PFC emulsion by macrophages was slowed down when an *F*-alkylated PEG surfactant was used as the emulsifier (Hsu and Peng, 2001). A PFOB emulsion stabilized by phospholipids and an *F*-surfactant, and surface-modified with a distearoylphosphatidylethanolamine-PEG, was demonstrated to deliver O_2 in a cardiopulmonary bypass with hemodilution canine model (Isaka *et al.*, 2003).

Exchange transfusion with a PFC emulsion helped reduce the hemoglobin signal in a study of the functional organization of the brain, using optical signals evoked by peripheral nerve simulation in rat cortex (Nomura *et al.*, 2000). An emulsion of *F*-15-crown-5-ether (20 magnetically equivalent fluorines) and ^{19}F magnetic resonance imaging (MRI), used as a 'gold standard' allowed validation of an ^{1}H MRI method to quantitate changes in tumor oxygenation in carbogen (95% O_2/5% CO_2)-breathing rats (Fan *et al.*, 2002). Assays that allow detection of PFCs in blood by headspace solid-phase microextraction combined with gas chromatography/mass spectrometry have been developed as part of anti-doping in sport efforts (Mathurin *et al.*, 2001). The benefits of PFCs and PFC emulsions to cell cultures have been reviewed (Lowe, 2002). Reverse, i.e., water-in-PFC emulsions, gel-emulsions and PFC microemulsions are also being investigated (Krafft *et al.*, 2003).

Substantial efforts are currently being devoted to investigating targeted PFC emulsions for the purpose of molecular imaging – i.e., detection of molecular markers, such as proteins and other cell-surface receptors, characteristic of a given pathology (Lanza and Wickline, 2001; Lanza *et al.*, 2002; Marsh *et al.*, 2002; Riess, 2004). Such emulsions also have potential for site-directed drug delivery and monitoring of therapy. Important potential target pathologies include inflammation, atherosclerosis, tumor-related angiogenesis, and thrombi. Detection and differentiation from normal tissue involves binding ligands specific for epitopes of interest onto the emulsion particles. Incorporation of paramagnetic material, e.g. a gadolinium complex, into the lipid monolayer of such targeted emulsion droplets provides contrast agents useful for both ultrasound and magnetic resonance imaging.

EDITOR'S SUMMARY

Perfluorocarbons (PFCs) are completely synthetic materials in which most hydrogen atoms are replaced by fluorine. Besides their synthetic source, the chief property of perfluorocarbons that makes them attractive as therapeutic agents is their capacity to dissolve gases in very large quantities.

In order for PFCs to be used clinically they must be emulsified, because the PFCs themselves are totally inert and are therefore

unable to interact with water – a requirement for aqueous solution. PFCs for clinical use are emulsified with materials that are lipophilic; these emulsifiers form tiny particles that include the emulsifier and the PFC.

The way in which PFC emulsions carry and release oxygen is fundamentally different from the way red blood cells, or even cell-free hemoglobin, carry and release oxygen. In the case of the PFC the oxygen content is a linear function of PO_2, whereas for hemoglobin the oxygen is bound through a chemical bond and not released until the PO_2 falls to physiologic levels. In spite of this difference, PFC emulsions have been shown to be capable of oxygenating tissues.

The creation of small particles of stable size has presented a considerable challenge to chemists working in the field, since on storage the particles tend to grow in size. Additional problems that have been addressed are the tendency to be engulfed by macrophages, with disparate consequences such as the release of inflammatory cytokines and falling platelet counts in some instances.

This chapter lays the groundwork for the several chapters that follow, in that it reviews relevant PFC chemistry and many of the more common PFCs that have been used in research leading to the development of clinical products.

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Chapter 25

Fluosol®: The First Commercial Injectable Perfluorocarbon Oxygen Carrier

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INTRODUCTION

In March 1979, a landmark in the development of synthetic 'blood substitutes' was reached when the Green Cross Corporation of Osaka, Japan, reported that ten of its employees had 'volunteered' to be injected intravenously with 20–500 ml of the world's first commercial emulsion of perfluorocarbons (PFCs), marketed as Fluosol-DA® (Ohyanagi *et al.*, 1979). This was the first step in the clinical safety evaluation of the emulsion as an injectable oxygen-carrier, which led to it becoming the first and, to date, the only such fluid to be approved for clinical use by the United States Federal Drug Administration (FDA) Committee (Table 25.1). As discussed by Jean Riess in Chapter 24 of this book, PFC liquids are colorless, odorless liquids that can dissolve large volumes of respiratory gases, principally oxygen. PFC liquids are also extremely hydrophobic and immiscible with blood and other body fluids. Consequently, for intravascular injection and use as tissue oxygenation fluids, PFCs must be prepared as fine emulsions, with a target droplet diameter of 200 nm or less.

This chapter gives an overview of the origins, chemistry, composition and production of Fluosol-DA (later Fluosol®), and describes preclinical and clinical studies with the emulsion that led to its regulatory approval as an oxygen-carrying fluid and adjunct to coronary angioplasty. Brief consideration is also given to some other applications of Fluosol as an organ/tissue perfusate and anti-cancer agent. Overall, Fluosol provided a key benchmark for the subsequent development of improved, more concentrated and room temperature stable 'second-generation' formulations of emulsified PFCs as injectable 'oxygen therapeutics', discussed elsewhere in this book.

ORIGINS OF FLUOSOL

The foundations for the development of injectable PFC emulsions on a large scale were laid by the pioneering experiments of Henry Sloviter and Toshiharu Kamimoto (1967), who described the use of emulsified FX-80 – predominantly *F*-(2-*n*-butyltetrahydrofuran) – for perfusing the isolated rat brain. Subsequently, Robert Geyer and colleagues at Harvard reported experiments in which virtually all of the blood of oxygen-breathing rats was replaced with an emulsion of perfluorotributylamine, $(C_4F_9)_3N$ (Geyer *et al.*, 1968). This and related work was summarized in the first Symposium on Inert Organic Liquids for Biological Oxygen Transport, held in Atlantic City, New Jersey (Various, 1970). The primary challenge of research in this emerging and exciting field was the production of a stable and heat-sterilizable,

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Table 25.1 Fluosol® a clinical chronology

Date(s)	Development	Reference(s)
1970s	Emulsion development and characterization	Naito and Yokoyama (1978)
1978	First use in human (decerebrate) patients	Makowski *et al.* (1979)
1979	Safety assessment in human volunteers	Ohyanagi *et al.* (1979)
1970s–1980s	Preclinical evaluation in severe anemia	
1980s	Clinical evaluation in severe anemia	Mitsuno *et al.* (1982) Tremper *et al.* (1982) Waxman *et al.* (1984) Karn *et al.* (1985) Mitsuno and Ohyanagi (1985) Stefaniszyn *et al.* (1985) Gould *et al.* (1986) Ohyanagi and Saitoh (1986) Spence *et al.* (1990, 1992)
1983	FDA rejection as treatment for anemia	
1980s	Preclinical evaluation as adjunct to PTCA	Rude *et al.* (1984) Roberts *et al.* (1986) Virmani *et al.* (1988)
1980s	Clinical trials as adjunct to PTCA	Anderson *et al.* (1985) Jaffe *et al.* (1988) Bell *et al.* (1990) Kent *et al.* (1990) Young *et al.* (1990) Forman *et al.* (1991)
1989	FDA approval as adjunct to PTCA	
1990–1994	Clinical use as adjunct to PTCA	
1994	Withdrawal of emulsion from market; production ceases	

physiologically acceptable emulsion with low droplet size (*ca*. 200 nm diameter or less) based on PFCs that were excreted from the body rapidly (*ca*. 14–21 days or less) after injection. The Green Cross Corporation (Osaka, Japan) took up the challenge when they embarked on the production of Fluosol on a commercial scale, with the ambitious objective of creating the world's first so-called 'artificial blood'.

CHEMISTRY, COMPOSITION AND PRODUCTION

The composition of Fluosol and the functions of its components are listed in Table 25.2. The principal PFC and oxygen carrier in Fluosol was perfluorodecalin ($C_{10}F_{18}$), a compound subsequently used widely in biological systems (Zuck and Riess, 1994; Lowe, 1999, 2003; Riess, 2000, 2001). Lesser quantities of a second PFC, perfluoro-*n*-tripropylamine, $(C_3F_7)_3N$, were added to stabilize the emulsion against droplet growth by the process of molecular diffusion known as Ostwald ripening (see Chapter 24). The total PFC content of the Fluosol emulsion was 20 per cent (w/v). Another, more concentrated (35 per cent w/v) version of Fluosol (known as Fluosol-DA 35 per cent) was produced for preclinical studies only (Naito and Yokoyama, 1978). The emulsifier (surfactant) components of Fluosol, essential stabilizing constituents of any emulsion system, were poloxamer 188, a polyoxyethylene-polyoxypropylene block co-polymer available commercially as Pluronic® F-68, egg yolk phospholipids (EYP) and potassium oleate (Table 25.2). The pH of Fluosol was adjusted to 7.3 and its osmolarity to 410 mOsm/l using mineral salts and dextrose (Riess, 2001), although early formulations contained glucose and hydroxyethylstarch to provide additional oncotic pressure (Lowe, 1986, 1987; Riess, 2001). Fluosol was manufactured on a commercial scale by high-pressure homogenization using a Manton–Gaulin apparatus, which was preferred over the more vigorous sonication process that typically led to the generation

Table 25.2 Composition of Fluosol®

Constituent	Concentration (w/v %)	Function
Perfluorodecalin	14.0	Oxygen carrier
Perfluorotripropylamine	6.0	Oxygen carrier
Pluronic® F-68	2.72	Emulsifier
Yolk phospholipids	0.40	Emulsifier
Potassium oleate	0.032	Emulsifier
Glycerol	0.8	Cryoprotectant
NaCl	0.6	Ionic balance, pH, osmotic pressure
$NaHCO_3$	0.21	Ionic balance, pH, osmotic pressure
Glucose	0.18	Ionic balance, pH, osmotic pressure
$MgCl_2$	0.043	Ionic balance, pH, osmotic pressure
$CaCl_2$	0.036	Ionic balance, pH, osmotic pressure
KCl	0.034	Ionic balance, pH, osmotic pressure

of fluoride ions from the PFCs (Riess and Le Blanc, 1982). The resulting average droplet size was approximately 200 nm or less, but the uniformity of size was uncertain. Because emulsions of perfluorodecalin are inherently unstable (Riess, 2001), the stem emulsion (400 ml) of Fluosol (PFCs and emulsifiers) had to be stored frozen. Thus, glycerol was added as a cryoprotectant (Table 25.2). The emulsion was reconstituted by careful thawing followed by the addition of the remaining constituents that had been stored separately as two annex solutions (with a total volume of 100 ml) under refrigeration. It was recommended that the reconstituted product (500 ml) be used within 8 hours.

PRECLINICAL STUDIES

Extensive preclinical studies were performed with Fluosol, focusing primarily on its intravascular dwell time, the tissue uptake and body retention time of its constituent PFCs, and its efficacy of oxygen transport under conditions of extreme anemia. In the early- to mid-1980s, there were reports of so-called 'bloodless' animals in which the blood of conscious rats had been exchange-transfused for Fluosol (Lowe *et al.*, 1982, 1985; Hardy *et al.*, 1983). Such animals provided a valuable experimental system for studying aspects of homeostasis under virtually bloodless conditions.

The body retention half-times in rats of the two PFCs in Fluosol (perfluorodecalin and perfluorotripropylamine), were approximately 7 days and 65 days, respectively (Naito and Yokoyama, 1978; Riess and LeBlanc, 1982). This difference was ascribed to the presence of an N_2 atom in the latter molecule, giving a prolonged retention time in tissues. PFC droplets are removed from the circulation by cells of the macrophage-monocyte (reticuloendothelial) system (MMS), giving cells of some organs (e.g. liver, spleen) a characteristic 'foamy' appearance (Nannay *et al.*, 1984). Following the infusion of large doses of Fluosol, tissue accumulation of PFCs typically caused enlargement of liver, spleen and other tissues, coupled with transient elevations in liver enzymes, generally in proportion to the dose administered (Lowe, 1988, 1994; Ravis *et al.*, 1991). This was often associated with altered immune system function, but the responses observed were highly variable, depending on species used and timing of emulsion administration relative to immune challenge (Lowe, 1988, 1994). The PFCs themselves were not metabolized in the body, and were eventually excreted primarily by exhalation through the lungs.

CLINICAL TRIALS

Studies in Japan

Fluosol was tested in clinical trials, initially in Japan and subsequently in North America (Table 25.1). Honda *et al.* (1980) reported the first use of Fluosol in one patient that had suffered a severe gastrointestinal bleed. An infusion of 500 ml of Fluosol was given with no recorded adverse effects prior to the administration of a blood transfusion. Mitsuno *et al.* (1981) also reported the infusion of 1000 ml of Fluosol in a patient

who suffered a major blood loss (975 ml) whilst undergoing surgery for esophageal cancer. Transient alterations in hematological variables, especially thrombocytopenia, were observed in the recipient postoperatively. Mitsuno *et al.* (1982) subsequently reported that 186 patients in Japan had been infused with Fluosol (30 ml/kg body weight or 6 g PFC per kg) for a variety of indications, including hemorrhage and chronic anemia. Beneficial effects on both hemodynamic indices and blood oxygenation were emphasized. Only minor adverse reactions, again including transient thrombocytopenia, were described. Unfortunately, no control group was included in the studies. Later, Ohyanagi and Saitoh (1986) reported that over 400 Japanese patients, all of whom were Jehovah's Witnesses who refused a blood transfusion, had been infused with the emulsion (Table 25.1). In these trials, the PFC and plasma phase oxygen contents increased but the overall oxygen content was unchanged. Hemodynamic variables remained stable, and no adverse reactions were reported. Importantly, recipients were also given an initial intravenous test dose of 0.5 ml of Fluosol to identify any acute adverse anaphylactic reaction. This became a routine recommendation to physicians prior to the infusion of larger doses of the emulsion to patients.

Studies in North America

The first person to receive Fluosol in the United States was a 67-year-old Jehovah's Witness from Minnesota with severe peripheral vascular disease and anemia (Vercellotti *et al.*, 1982). The patient had extensive bleeding from a surgical wound, and his blood hemoglobin had fallen to 3.8 g/100 ml. He became lethargic and disoriented, but refused a blood transfusion. He was therefore infused with 30 ml Fluosol over 15 minutes to improve blood oxygenation. The infusion of the emulsion was stopped, however, because of chest pain, shortage of breath and tachycardia. The patient's pulmonary artery pressure increased by 54 per cent. Antihistamines and corticosteroids were administered intravenously, with subsequent stabilization of vital signs.

More extensive experience of using Fluosol in severely anemic (hemoglobin: 1.9–7.5 g/dl) patients in the USA was described by Tremper *et al.* (1982) in a study involving 13 patients, of which data were collected from 7. The aim of the trial was to determine the clinical safety of the emulsion and its effects on oxygen transport and hemodynamic variables. Overall, tissue oxygenation increased significantly provided that patients breathed supplementary oxygen, effectively raising the fraction of inspired oxygen (F_IO_2) to 1 (100% O_2). This maximized oxygen loading onto the droplets of Fluosol in the lungs. Whilst the outcomes of the study were generally favorable, the incidence of adverse reactions was greater than had been seen in the Japanese trials. In this respect, two of the seven patients studied exhibited a symptomatic reaction to the test dose of Fluosol and it was speculated that this involved activation of the plasma complement cascade, as discussed later. Subsequent clinical studies in the USA included further trials in anemic surgical patients (Waxman *et al.*, 1984; Police *et al.*, 1985), while in related investigations Karn *et al.* (1985) used Fluosol in two patients for the treatment of major post-partum hemorrhage (Table 25.1). Some investigators speculated that Fluosol enhanced oxygen distribution within the tissue microcirculation – an effect maximized when patients' breathed supplementary oxygen (Tremper *et al.*, 1982; Waxman *et al.*, 1984). Police *et al.* (1985) described the use of Fluosol to treat three patients suffering from life-threatening hemorrhage. All the patients exhibited post-treatment complications of fever and respiratory complications. In one individual, the complications were fatal. The authors' suggested that the adverse reactions were caused by the emulsion droplets causing complement activation, coupled with the possible toxic effects of breathing 100% oxygen for prolonged periods.

It is now known that intravascular infusion of a PFC emulsion can, in some individuals, provoke the occurrence of flu-like symptoms, characterized by fever, chills and nausea (Riess, 2001). Such effects are the natural consequence of the clearance by the MMS of particulate matter (in this case, PFC emulsion droplets) from the bloodstream. Phagocytosis is accompanied by the release of products from the arachidonic cascade, especially cytokines and prostaglandins, causing the flu-like reactions and respiratory and hemodynamic perturbations.

One interesting application of Fluosol was in the management of a trochanteric pressure sore in an anemic Jehovah's Witness patient. The patient received an intravascular infusion of 1500 ml of Fluosol over a 5-hour period to elevate arterial oxygen tension, followed by successful reconstructive surgery (Brown *et al.*, 1984).

In Canada, Fluosol was infused into three anemic patients in anticipation of operative blood loss (Stefaniszyn *et al.*, 1985). The results were inconclusive, but the authors concluded that *Fluosol* probably did contribute a significant proportion of the consumed oxygen at high F_IO_2. They highlighted the occurrence of adverse reactions to the 0.5-ml test dose of the emulsion that occurred in two of the three patients studied.

Perhaps the most controversial clinical study was the prospective, non-randomized trial using Fluosol in eight severely anemic Jehovah's Witness patients (hemoglobin: *ca.* 3.0 g/dl or less) who had refused a blood transfusion (Gould *et al.*, 1986). Infusion of Fluosol (40 ml/kg) increased both oxygen delivery and consumption, but the authors' concluded that this was insufficient to improve patient survival. Emphasis was placed on the low concentration and relatively short intravascular dwell time of the PFCs in Fluosol, as discussed already, together with the limited volumes of the emulsion permitted to be infused under the study protocol. This issue was hotly contested by other investigators who criticized the conclusions drawn from the investigation, emphasizing (1) the low numbers of 'highly select' patients, studied without a control group; and (2) the mortality of patients with blood hemoglobin below 3.5 g/100 ml, in the absence of any treatment with Fluosol, normally being extremely high (Hermoni *et al.*, 1986; Spence *et al.*, 1986). Nevertheless, the study of Gould *et al.* (1986) was subsequently often cited as providing proof of a lack of efficacy of Fluosol for improving the survival of anemic patients without blood transfusion.

A randomized clinical trial of Fluosol involving 46 patients was published in 1990 (Spence *et al.*, 1990), with a follow-up study with 52 patients appearing 2 years later (Spence *et al.*, 1992). Again, the patients were Jehovah's Witnesses with severe anemia (mean hemoglobin: 4.6 g/100 ml) following acute blood loss. Infusion of Fluosol (30 ml/kg) stabilized the patients and increased the dissolved or plasma oxygen content at 12 hours after infusion, but not the overall oxygen content when compared to control patients receiving crystalloid solution. The infusion protocol was not continued with four patients who showed an adverse reaction to the 0.5 ml test dose of the emulsion. The conclusions from these studies were that a low volume of 'dilute' emulsion (Table 25.3) had only minimal beneficial effects on oxygen delivery and that this did not persist beyond about 12 hours owing to the short intravascular dwell time of the product.

Table 25.3 Positive and negative features of Fluosol®

Positive
- 'Proof of principle' for intravascular oxygen delivery using perfluorocarbons
- Preclinical and clinical data on *in vivo* behaviour of perfluorocarbons
- Baseline product for development of improved, more concentrated, injectable emulsions

Negative
- 'Dilute' emulsion
- Poor stability
- Need to ship and store stem emulsion frozen
- Cumbersome, multi-step reconstitution procedure
- Low intravascular persistence time
- Prolonged body retention of perfluorocarbon constituents
- Adverse reactions to emulsion in some patients
- Concerns about toxicity of Pluronic® F-68 surfactant in some patients

FAILURE OF REGULATORY APPROVAL OF FLUOSOL FOR THE TREATMENT OF ANEMIA

In 1983, the US FDA rejected a new drug application filed by the Green Cross Corporation for the use of Fluosol as clinical therapeutic treatment for anemia (Table 25.1). The decision was based on unproven efficacy in affecting clinical outcome. Not surprisingly, this outcome was based on the short intravascular persistence time (*ca.* 6–12 hours) of the product, although the safety of the emulsion and its ability to transport oxygen were not questioned (Marwick, 1983). The shortcomings of using Fluosol for treating anemic patients are listed in Table 25.3, and were subsequently discussed at length in keynote reviews (Zuck and Riess, 1994; Keipert, 1998; Lowe, 1999, 2003; Riess, 2000, 2001).

PERCUTANEOUS TRANSLUMINAL CORONARY ANGIOPLASTY

What is angioplasty?

As noted already, Fluosol remains to this day the only PFC-based oxygen therapeutic to be approved for clinical use in the USA (Table 25.1).

The specific indication for which the emulsion was licensed was as an oxygen-carrying, low viscosity intracoronary perfusate during percutaneous transluminal coronary angioplasty (PTCA) or, more simply, angioplasty. The latter procedure, first performed by the late Andreas Gruentzig and his Swiss colleagues in September 1977 (Gruentzig, 1978), has become a primary intervention for the treatment of coronary heart disease. PTCA is used therapeutically to open a partially occluded, atherosclerotic coronary artery, thereby alleviating the symptoms of ischemia, such as angina. The procedure involves the insertion and inflation of a balloon catheter inside the coronary vessel to crush the atheroma plaque, thereby improving blood flow. One common problem with early trials using angioplasty was an exacerbation of ischemia distal to the balloon, caused by oxygen deprivation. This problem could be solved by the simultaneous infusion of an oxygen-carrying fluid (Kerins, 1994; Ogilby, 1994). Blood was, of course, an obvious choice, but it was initially difficult to achieve the desired perfusion rates without causing red cell lysis and related complications.

Benefits of Fluosol in angioplasty: animal and clinical studies

Initial studies in animals showed that intra-arterial infusion of Fluosol administered with a power injector was highly effective at preserving both global and regional ventricular function, including a decrease in the appearance of abnormal electrocardiographic (ST segment) changes, during balloon inflation (Rude *et al.*, 1984; Roberts *et al.*, 1986; Virmani *et al.*, 1988; Table 25.1). Subsequent clinical studies assessed the efficacy of intracoronary infusion of warmed, oxygenated Fluosol. For example, Anderson *et al.* (1985) reported that Fluosol infusion during a 90-second balloon inflation lengthened the time to angina, shortened the duration of angina and lessened the degree of ST segment changes, compared to control patients infused with lactated Ringer's solution. In a subsequent large, multicenter study involving 245 patients, it was reported that in individuals subjected to angioplasty with simultaneous transcatheter infusion of oxygenated Fluosol at 60 ml/min, the incidence of severe angina was significantly lower than for patients receiving PTCA without Fluosol (Kent *et al.*, 1990). Both global and regional ventricular function were preserved in the Fluosol treatment group, but not in controls. The authors' concluded that the emulsion was highly effective in alleviating myocardial ischemia during angioplasty. Similar general conclusions were reached in other clinical trials of Fluosol in PTCA (Bell *et al.*, 1990; Young *et al.*, 1990; Forman *et al.*, 1991). It was argued at the time that the specific properties of Fluosol, including the ability to carry oxygen, the low viscosity and the small droplet size, made the emulsion ideal for augmenting the effectiveness of angioplasty.

Regulatory approval of Fluosol for clinical use as adjunct to angioplasty

As mentioned already, this led to a landmark decision, in December 1989, by the United States FDA Committee (Anon, 1990), to approve Fluosol for use during PTCA in high-risk patients. Following approval by the regulatory authorities, the wholly owned US subsidiary of Green Cross, Alpha Therapeutic Corporation, supplied Fluosol for use in over 13 000 patients per year for 3 years. However, about half of the uses of the emulsion were off-license as an alternative to transfusion of blood, with the remainder used to augment PTCA. In the UK, Fluosol was marketed by Alpha Therapeutic UK Ltd, Thetford, and the emulsion was designated the product licence number PL4447/0015. Reports of the first use of the emulsion in British patients by Dr Pitts-Crick and his colleagues at Bristol Royal Infirmary appeared in the media in early 1991. The specific application and benefits of using Fluosol as an adjunct to angioplasty were subsequently described in several reviews (Kerins, 1994; Klonar and Hale, 1994; Ogilby, 1994). In early 1994, however, Green Cross ceased manufacturing Fluosol due to lack of interest from physicians and poor sales in their angioplasty indication (Table 25.1). Improvements in PTCA technology, notably the introduction of specialized autoperfusion catheters, made the need for Fluosol redundant. Such catheters were initially developed as devices to improve blood flow to the distal coronary bed in cases of failed angioplasty requiring emergency coronary artery bypass surgery (Kerins, 1994). One early catheter had multiple holes, enabling blood to enter the catheter in the proximal region, flow through the catheter in the area of vessel occlusion and exit in the distal region (Hinohara *et al.*, 1988). An improved autoperfusion catheter, with a central inflatable balloon, enabled effective angioplasty to be performed without the need for the

simultaneous infusion of an oxygenation fluid (Quigley *et al.*, 1998). With the development of modern angioplasty catheters that did not permit the distal infusion of Fluosol (Ogilby, 1994), it was perhaps not surprising that physicians did not use the product – leading, eventually, to its withdrawal from the market (Table 25.1).

OTHER APPLICATIONS FOR FLUOSOL

In addition to the applications already described, Fluosol was used for preserving traumatically excised human extremities (mainly finger digits) for up to 72 hours prior, in some cases, to their successful replantation (Smith *et al.*, 1985). The emulsion was also evaluated for physiological investigation and preservation of both animal and human organs, including the heart and lung (Lowe, 1997). Indeed, Fuchinoue *et al.* (1986) reported the successful re-implantation of human kidneys following their *ex vitro* perfusion with Fluosol.

Fluosol was also studied as an adjunct to cancer therapy. Solid tumors contain hypoxic cells, and this can impair the efficiency of many anti-cancer agents, including ionizing radiation and chemotherapeutic drugs, which depend on oxygen to be effective. Extensive preclinical studies evaluated the effectiveness of Fluosol with oxygen or carbogen (95% oxygen, 5% carbon dioxide) breathing in conjunction with ionizing radiation as an anti-cancer therapy, with generally positive outcomes (Teicher, 1992, 1995; Rockwell, 1994). Additional beneficial effects of this treatment regimen with radiosensitizing drugs, including nicotinamide, which improves microregional blood flow (Horsman, 1995), were similarly effective (Teicher, 1995). Related preclinical investigations reported increased tumor cell killing by chemotherapeutic drugs, including cyclophosphamide, when used in conjunction with Fluosol and carbogen (Teicher, 1994).

In 1989, Lustig and others described the results of a Phase I/II clinical study evaluating Fluosol and 100% oxygen breathing as an adjuvant to radiation for the treatment of advanced squamous cell tumours of the head and neck (Lustig *et al.*, 1989). The study was an extension of a trial initiated in 1984, with initial results being reported by Rose *et al.* (1986). One aim of the investigation of Lustig *et al.* (1989) was to determine the maximal weekly tolerable dose of the emulsion. Patients were infused weekly with Fluosol in an escalating dose regimen with a maximum of 56 ml/kg in eight individuals, and then breathed 100% oxygen for a minimum of 30 minutes prior to and during radiation exposure. The absolute survival of patients at 1 year following treatment was 67 per cent. Acute reactions to a 0.5 ml test dose of the emulsion occurred in 11 per cent of recipients, with the responses being consistent with activation of complement, as discussed later. Similar mild to moderate adverse reactions to test dose of Fluosol were reported in a Phase I/II clinical study by the same research team in patients with lung carcinoma (Lustig *et al.*, 1990). Teicher (1992, 1995) subsequently described the context and outcomes of these clinical trials more fully.

In 1987, a Phase I/II trial was initiated to determine the toxicity and efficacy of Fluosol with 100% oxygen as an adjuvant to radiation for the treatment of high-grade brain tumors (Evans *et al.*, 1990, 1993). The mean survival time following treatment in patients receiving Fluosol was 75 weeks, which was not significantly different from a corresponding value of 54 weeks for a matched control group. However, the results showed that Fluosol was beneficial in patients who survived more than 1 year after treatment. Importantly, the studies also indicated that the Fluosol/oxygen treatment regimen sensitized hypoxic cells in the brain to the cytotoxic effects of the radiation therapy.

Dowling *et al.* (1992) reported the results of the first clinical feasibility study on the use of Fluosol and hyperbaric oxygen (3 atmospheres) as an adjunct to radiotherapy (600 cGy) in 20 patients with malignant gliomas. The dose of Fluosol administered was escalated from 42 ml/kg in six courses to 80 ml/kg in four courses. There was, however, no significant improvement in patient survival with the Fluosol treatment, and the manufacturer terminated the trial when the total dose of emulsion administered reached the maximum allowable.

In 1994, the findings were published of an extensive, multicenter clinical study (TAMI-9), involving 430 patients, to assess the safety and efficacy of Fluosol as an adjunct reperfusion therapy following treatment with the thrombolytic agent, tissue-type plasminogen activator (TPA; Wall *et al.*, 1994). The outcomes of the study were disappointing since, when given with TPA, Fluosol did not improve ventricular systolic function, reduce infarct size or improve the overall clinical outcome.

LESSONS LEARNED FROM FLUOSOL

In his comprehensive review of the field of 'blood substitutes', Jean Riess discussed the merits and shortcomings of Fluosol, emphasizing the reasons why it was not adopted by the clinical community (Riess, 2001). Table 25.3 summarizes the principal positive and negative features of Fluosol in relation to its clinical use as an intravascular oxygen carrier, or so-called 'oxygen therapeutic'. The latter term is now preferred for PFC- and hemoglobin-based fluids rather than 'blood substitute', although the term is used purposely in the title of this book because, as noted in the Preface, they are designed to be used as alternatives to transfusions of red blood cells.

Merits

Fluosol, being the first commercial and well-characterized emulsion to be widely available for extensive preclinical and clinical studies, was used to demonstrate 'proof of principle' that PFCs had merits as injectable tissue oxygenation agents. The extensive biological studies performed with Fluosol over almost a 20-year period, in many laboratories throughout the world, contributed hugely to the understanding of the behavior and benefits of PFCs and their emulsions *in vivo* and *in vitro*. Such information has been reviewed extensively, and will not be repeated here (Zuck and Riess, 1994; Keipert, 1998; Lowe, 1999, 2003; Riess, 2000, 2001). Experience with Fluosol was pivotal in informing the development of improved, more concentrated, room temperature stable 'second-generation' injectable PFC emulsions, such as the Alliance Product, Oxygent™ (see Chapter 28).

Shortcomings

The negative features of Fluosol, in terms of (1) it being a 'dilute' formulation (i.e., low PFC content) with poor stability necessitating the need for the stem emulsion to be to shipped and stored frozen, (2) the cumbersome, multi-step reconstitution procedure, (3) the low intravascular persistence time of the product, and (4) the prolonged tissue retention time of the perfluorotripropylamine constituent, have been discussed more thoroughly earlier in this chapter.

Adverse anaphylactoid reactions to Fluosol: problems with the Pluronic surfactant

Following the reports from the initial clinical studies with Fluosol of adverse reactions to a small test dose in some patients (e.g. Tremper *et al.*, 1982), an intensive research effort was focused on elucidating the reasons for and underlying mechanism(s) of these effects. Greg Vercellotti and Dale Hammerschmidt demonstrated that Fluosol was effective in causing activation of the plasma complement system *in vitro* by the alternative pathway (Vercellotti *et al.*, 1982; Vercellotti and Hammerschmidt, 1988). They speculated that this was responsible for the acute, transient anaphylactoid reactions observed in some patients receiving the emulsion. Importantly, their experiments also revealed that the principal surfactant component of Fluosol, Pluronic F-68 (Table 25.2), could similarly induce activation of complement (Vercellotti *et al.*, 1982; Vercellotti and Hammerschmidt, 1988). These findings provoked significant debate on the applicability of findings obtained in *in vitro* test systems to *in vivo* clinical situations.

Subsequent intensive research reviewed by Lowe (1987, 1994) revealed that many of the adverse physiological effects of Fluosol, including alterations in blood leukocyte and vascular endothelial cell functions, could be attributed to Pluronic F-68. Others (McCoy *et al.*, 1984) speculated that peroxide derivatives of this compound, formed during steam sterilization of long-term storage of the emulsion, contributed to such effects. Concerns over the toxicity of Pluronic prompted a move towards the development of phospholipid-stabilized emulsions that did not cause such profound adverse effects in recipients (Riess, 2001; Lowe, 2003). Again the reader is referred to Chapter 28, describing the development of the commercial Oxygent emulsion, in which EYP are used as surfactant to improve biocompatibility.

OTHER 'FIRST-GENERATION' EMULSIONS

The development and evaluation of Fluosol was paralleled by the production and testing of other 'first-generation' injectable PFC emulsions. Three such emulsions and their countries of origin are:

- Emulsion No. II (China)
- Oxypherol® (Japan)
- Perftoran® (Russia).

Emulsion No. II contained perfluorodecalin and perfluorotripropylamine, with a Pluronic F-68 surfactant, and had an average droplet diameter of *ca.* 200 nm. It was thus broadly similar to *Fluosol*®. The emulsion was produced by the Institute for Organic Chemistry, Shanghai. It was reported that Emulsion No. II was administered to several hundred patients, including war casualties (Xiong *et al.*, 1981; Chen and Yang, 1989), but little further information on this product is available.

Oxypherol® (formerly Fluosol-43) was an additional, highly stable emulsion produced commercially by Green Cross. The emulsion contained 20 per cent (w/v) perfluorotributylamine stabilized with Pluronic F-68. Oxypherol was widely used in animal studies and for the perfusion of isolated organs, but was not advocated for human use because of the unacceptably long retention half-time (>500 days) of this compound in body tissues (Naito and Yokoyama, 1978; Zuck and Riess, 1994; Lowe, 1997, 2003; Riess, 2000, 2001).

Perftoran® (formerly Ftorosan) contains 7.6 per cent (v/v) perfluorodecalin and 3.8 per cent (v/v) perfluoro *N*-(4-methylcyclohexyl)piperidine ($C_{12}F_{22}N$) stabilized with a poloxamer-type (Proxanol 258) surfactant (Beloyartsev *et al.*, 1983; Golubev, 1998). The emulsion is filter sterilized and is stable for about 30 days under refrigeration. The emulsion was developed jointly by the Russian Academy of Sciences of the Perftoran Company (Pushchino, Russia) and licensed by the Ministry of Health of the Russian Federation in 1997 for a range of indications, principally as an anti-ischemic agent and *ex vitro* perfusate for organs for transplantation. Perftoran is distributed through clinical institutions, and an emergency 'bank' of the emulsion has been created. At the time of writing, Perftoran remains the only oxygen therapeutic to be licensed for clinical use. A translation of an overview of the production and biomedical applications of PFCs in Russia has been published (Ivanitsky, 2001), and discussion of the current status of Perftoran by Eugene Maevsky follows in Chapter 26.

EDITOR'S SUMMARY

Fluosol-DA (Fluosol) was the first injectable oxygen carrier approved by the US FDA for clinical use. As a result, extensive preclinical and clinical data are available for review, and much of the experience is directly applicable to the development of subsequent formulations of both perfluorocarbon- and hemoglobin-based blood substitutes.

Preclinical administration of Fluosol resulted in enlarged livers and spleens and foamy macrophages on histological examination of postmortem animal tissue samples. However, Fluosol was able to support life in the absence of red blood cells, so there was no question that it was able to transport oxygen.

Clinical trials, carried out in Japan, Canada and the US with doses ranging up to 500–1000 ml per patient, were encouraging, but there was a significant incidence of complement activation, fever and flu-like syndrome, which was attributed to activation of the monocyte/macrophage system. A critical study concluded that because of the dose limitation that these side effects impose, and the hemodilution that results from administration, no efficacy could be shown when Fluosol was used as a replacement for red blood cells.

Work continued in the development of Fluosol for local tissue oxygenation, specifically as an adjunct in coronary angioplasty. This led to its eventual approval by the FDA for this specific application. However, efficacy was marginal and the use of the product was cumbersome. Fluosol was eventually taken off the market by the manufacturer.

Although Fluosol was not a long-term success, the research led the way to improved formulations that had higher oxygen capacity and fewer side effects.

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Chapter 26

Perftoran®

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INTRODUCTION

Perftoran was developed in the Institute of Theoretical and Experimental Biophysics of the Russian Academy of Sciences, and was registered in Russia in February 1996 (later in Ukraine and Kazakhstan) as an oxygen-carrying blood substitute manufactured by the Scientific Productive Company Perftoran (Ivanitsky, 2001). Perftoran is an emulsion of 10 volume per cent of perfluorochemicals (PFCs), the main two being perfluorodecaline (PFD) and perfluoro-N-4-(methylcyclohexyl)-piperidine (PFMCP) in the ratio 7:3 (Maevsky *et al.*, 2003). The intravascular retention half-time for Perftoran is 9 hours in rats and about 20 hours in rabbits. The half-times for removing PFD and PFMCP from the organism are about 2 and 13 weeks, respectively. PFC emulsion is stabilized by the 4 per cent polyoxyethylene-polyoxypropylene copolymer Proxanol P268, and contains a physiologically acceptable saline solution (Table 26.1). Perftoran is packed in 100-, 200- and 400-ml bottles, and can be stored for 3 years at −4°C––18°C, and for 2 weeks at 4°C.

Table 26.1 Composition of Perftoran® (from the Scientific-Productive Company 'Perftoran', Russia)

Perfluorodecalin and its co-products	7.0 ml
Perfluoro-N-(4-methylcyclohexyl)-piperidine and its co-products	3.0 ml
Proxanol P268	4.0 g
NaCl	0.6 g
KCl	0.039 g
$MgCl_2$	0.019 g
$NaHCO_3$	0.065 g
NaH_2PO_4	0.02 g
Glucose	0.2 g
H_2O	100 ml
$[F^-]$	10 μM
Osmotic pressure	300 mOsm
pH	7.3
Viscosity	2.3 cPs
Average particle size	60 nm

HISTORY AND COMPOSITION OF PERFTORAN

In the Soviet Union, the development of PFC-based oxygen carriers for blood replacement

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was initiated by Zoya Chaplygina and Grigory Rosenberg in the early 1970s. At that time the chemical school of the academician Knunyants provided a foundation for the manufacture of PFCs for military and civilian needs. The first PFC emulsions were developed independently by Irina Kuznetsova (Leningrad), Natalia Konovalova (Kupavna) and Dmitry Sidlyarov (Moscow). In 1979, Professor Felix Beloyartsev, together with the academician Knunyants and a corresponding member Ivanitsky, began to advance the multipurpose scientific studies of biomedical applications of PFCs. It was the starting point for the creation of Perftoran. In 1980, the efforts of different specialists of biomedical and technological institutes and enterprises were consolidated within the All-Union Scientific-Industrial Programme. An original PFC composition for Perftoran, including PFD and PFMCP, was suggested by Kiril Makarov and Lev Gervitz at the Institute of Elemental Organic Compounds.

Some Perftoran properties resemble those of Fluosol-DA, but we would like to note some differences between the two. As described in the Green Cross Technical Information (1978), the stem emulsion in Fluosol-DA contains PFD and perfluorotripropylamine (PFTPA), which are emulsified with Pluronic F68 and yolk phospholipids. However, the emulsion of PFD and PFMCP in Perftoran is stabilized only by Proxanol P268. Pluronic F-68 and Proxanol P268 are both copolymers, but it seems that Proxanol P268 is less toxic and more biocompatible than Pluronic F68. Moreover, the whole composition of Fluosol-DA includes an oncotic agent, 3 per cent hydroxyethylstarch, which can destroy the adsorption layer of emulsion particles. Perftoran does not contain any oncotic agents.

Regarding storage, the ready-for-use Perftoran is packed in a single bottle, while Fluosol-DA is presented as three separate solutions that must be mixed prior to use. Both the stem emulsion of Fluosol-DA and the whole Perftoran composition can be kept in a frozen state, but once Fluosol-DA has been thawed and mixed it can only be used for infusion for up to 8 hours, whereas defrosted Perftoran can be used for about 2 weeks.

As for side effects, the technology that was accepted in 1996 provided for a narrow distribution of emulsion particles with an average size of 0.06–0.07 μm, whereas the average particle size in Fluosol-DA is 0.12 μm. Owing to the smaller particle size (reduced from 0.12 to 0.07 μm in Perftoran), the frequency of side reactions with Perftoran has decreased from 8–10 per cent to 2–4 per cent (Vorobyev and Ivanitsky, 1997).

It is evident from the above mentioned differences that Peftoran is a more user-friendy drug than Fluosol-DA.

PRECLINICAL STUDIES

Preclinical experimental studies have shown that Perftoran is harmless and non-toxic for mice, rats, rabbits and dogs. The intraperitoneal median lethal dose (LD_{50}) is 239 ml/kg for adult mice and 183 ml/kg for young mice; the intravenous LD_{50} exceeds 140 ml/kg for rats (Rybalkin *et al.*, 2002). We did not observe any toxic symptoms, changes in body weight or food consumption, or significant shifts in hematological and biochemical parameters in the blood of rats for more than 6 months following intravenous administration of Perftoran (20 and 50 ml/kg). Repeated intravenous Perftoran infusions into rabbits, of 50 ml/kg each day for 3 consecutive days, were followed by a transient increase in the concentration of urine, bilirubin and cholesterol, as well as transaminase activities in the blood 24 hours after the last infusion. However, later and for 6 months these parameters returned to a normal level. Weight indexes of liver and spleen grew in proportion to the dose of Perftoran and normalized within the period of the PFC's retention time. Our composition of PFD with PFMCP enables complete elimination of toxicity in rabbits, which is an attribute of emulsions containing PFD (Sklifas *et al.*, 1993). Owing to its low lipophilicity PFMCP has a rather long half-time of retention in organs (90 days), and this led us to carry out thorough safety testing. Complete elimination of PFCs from organs was not followed by any side effects in tissue morphology or cell ultrastructure in the liver, spleen, lungs, bone marrow, kidneys and other organs (Vasiliev and Golubev, 1984). Perftoran and its components do not have any mutagenic or carcinogenic activity. Moreover, some of the malignant neoplasms among spontaneous tumors occurred 1.5 times less often in the group undergoing repeated Perftoran administrations than in the control group of intact animals. After 11 successive intraperitoneal Perftoran infusions (a total dose of 275 ml/kg) into pregnant rats during the first stage of pregnancy, symptoms of teratogenicity were found. Perftoran does not induce hypotensive reactions either in rats or in dogs, in contrast to Fluosol (Faithfull and Cain,

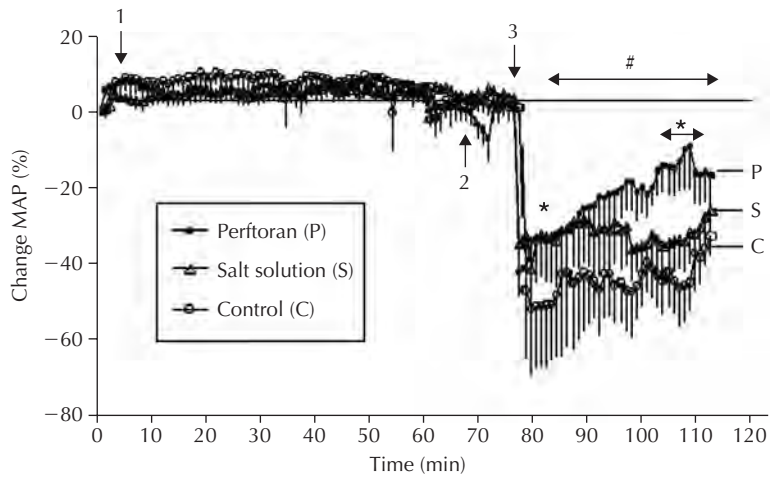

Figure 26.1 Comparison of Perftoran® and salt solution influences on the mean arterial blood pressure (MAP) in rats SHR-SP (the mean weight of the rats is 290 g). Arrow 1 – infusions of Perftoran or salt solution (the same salt content as in Perftoran) in doses of 7.5 ml into femoral vein through the preliminary inserted catheter; arrow 2 – taking 2 ml blood for examination of thrombocoagulation parameters; arrow 3 – taking 5 ml blood. MAP was measured with the electromanometer connected with a polyethylene catheter which was inserted into the femoral artery the day before the experimental procedure. Analogue signals of 512 cPs were sent from the electromanometer to the computer. #, comparison with the control level ($P < 0.05$), *, comparison with control or salt solution ($P < 0.05$). (From Tuhovskaya *et al*., 2004.)

1988). Furthermore, Perftoran infusion of 7.5 ml/kg before moderate blood loss maintains the mean arterial pressure (MAP) in rats better than salt solution (Figure 26.1). After massive blood replacement with Perftoran, the hematopoiesis in rats was activated in the usual way.

Because of the low PFC concentration and small oxygen capacity of Perftoran (6.9 ml/dl of oxygen at PO_2 760 mmHg), we estimated the oxygen-carrying efficiency of Perftoran by the function of rat liver mitochondria (RLM) after massive blood replacement in rats. The animals' survival was almost identical: 19 out of 20 with Perftoran, and 18 out of 20 with albumin-salt solution. However, their quality of life was different; the respiratory and phosphorylation activities of RLM were completely destroyed after isovolemic replacement with protein-salt solution, while with Perftoran (supplemented by 3% albumin) RLM retained the enhancement of phosphorylation and the respiratory rate as with autologous blood (Figure 26.2).

Onishenko *et al*. (1990) demonstrated better oxygen delivery by Perftoran, estimating the survival of kidney grafts isolated after hemorrhagic shock induced by an acute blood loss (35 ml/kg). The arterial blood pressure decreased from 150/70 to 50/30 mmHg, and the kidney blood flow dropped from 2000 ± 120 ml/min per kg to 800 ± 60 ml/min per kg. One hour later, the

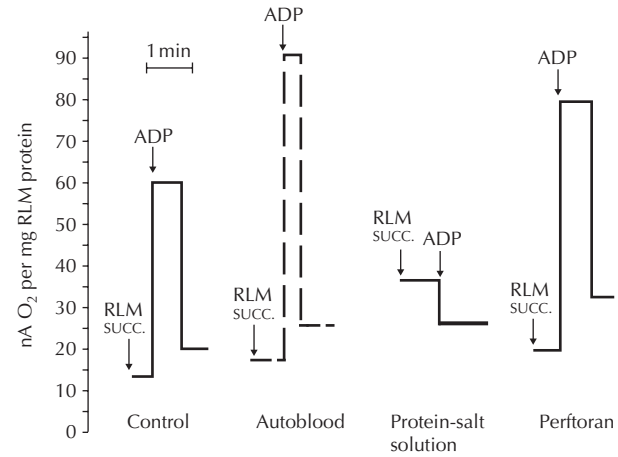

Figure 26.2 Diagrams of polarographic registration of respiratory rate of rat liver mitochondria (RLM) during oxidation of 5 mM potassium succinate (succ.) and phosphorylation of 150 μM ADP. Each curve is a mean value of six measurements (s.e.m. <10% of mean value). RLM were isolated in 6 h after blood replacement by scavenged autologous blood, protein-salt solution or by Perftoran (Hb content decreased from 16.0 ± 1.4 to 5.0 ± 0.6 g/dl in both groups). Incubation medium for RLM: 250 mM sucrose, 10 mM KCl, 10 mM Tris-HCl (pH 7.4), 3 mM KH_2PO_4, 3 mM $MgCl_2$, (RLM protein 2–3 mg/ml), t 26°C. After blood replacement by protein-salt solution or by Perftoran, animals were kept at PO_2 of 550–600 mmHg. (Based on data from Maevsky *et al*., 1999.)

bleeding dogs were treated with infusions of dextran 60 or Perftoran in the volume of about 40 ml/kg while breathing an oxygen-air mixture. After a 2-hour period of hemorrhagic shock, the kidneys were isolated from the 'hemorrhagic' animals and transplanted into recipient dogs that had previously undergone nephrectomy. The treatment of donor dogs with Perftoran enabled maintenance of ATP/ADP at a level two-fold higher, and decreased lactate/pyruvate ratios five-fold in the kidney tissue, in contrast to those in the dextran group. Correspondingly, the levels of creatinine and urea in the recipient blood serum were four-fold smaller and the kidney graft lifespan was twice as long if the donor dog was treated with Perftoran.

Sinchuk (1998) described similar shifts of hemodynamic and oxygen transport parameters in bleeding dogs treated with Perftoran or crystalloid solution (with subsequent dextran 60 infusions in both groups) 1–1.5 hours after blood losses of 25–45 ml/kg. He did not find any differences in oxygen regimes after plethoric infu-sion (30 ml/kg) of Perftoran or dextran 60 during acute lethal methemoglobinemia induced by nitrate poisoning. In this situation, 50 per cent isovolemic blood replacement with Perftoran had big advantages in comparison with dextran 60: cardiac output increased 1.5-fold ($P < 0.05$), arterial blood pressure was increased by 21 mmHg ($P < 0.05$), arterial PO_2 reached 270 mmHg, and the pH was 7.35. This compared with a PaO_2 of 92 mmHg and a pH of 7.27 with dextran 60 ($P < 0.05$).

We (Kuznetsova, 1997; Ivanitsky, 2001) suggest that an improvement in oxygen delivery by Perftoran is due to:

- the additional O_2 capacity of the PFC emulsion
- the faster O_2 and CO_2 consumption and release by the PFC emulsion
- enlarged gas gradients and diffusion surfaces
- a vasodilatation effect (probably connected with NO dissolution in PFCs).

Vasodilatation may be an important condition for providing oxygen delivery by even a very small quantity of erythrocytes (Winslow, 2003). Moreover, PFC particles can go through narrowed vessels that are impassable by erythrocytes, which are 70–100 times larger. Perftoran seemed to improve oxygen delivery together with the circulating erythrocytes, forming a reversible gas-carrying conveyer.

Due to a lipophilic relationship with biostructures, PFCs and their emulsions have a biological activity which is responsible for some unexpected effects: prolongation of cardioplegic preservation (Beloyartsev *et al.*, 1986), alterations in macrophage activities (Golubev, 1998), diminution of the secondary alteration during inflammation (Moiseenko, 1999; Orlov *et al.*, 2004), and a decrease in transplant rejection (Shumakov *et al.*, 1999). According to Islamov *et al.* (1986), when a cardioplegic medium contained Perftoran, both ischemic contracture and a decrease in pH in the tissue of isolated arrested hearts were delayed for at least twice as long. Reperfusion by Perftoran after total ischemia of isolated rabbit heart provided a two-fold higher resuscitation of contractility amplitude than that achieved with Tirode solution (Figure 26.3). The drug infusion in doses exceeding 2 ml/kg causes an induction of cytochrome P450 synthesis *de novo* and hence activates the monooxygenase system of the liver for the period of PFD retention (Obraztsov *et al.*, 1994).

CLINICAL TRIALS WITH PERFTORAN

During the three stages of preregistration clinical trials (1984–1994), Perftoran was administered intravascularly into 964 patients (Krylov *et al.*, 1985), in dosages ranging from 4 to 30 ml/kg depending on the disease (Table 26.2).

At massive blood replacement of more than 40 per cent of the circulating volume, Moroz *et al.* (1995, 1999) used Perftoran in doses of 1350–3600 ml, with the fraction of inhaled O_2 (F_IO_2) being 0.4–0.6. When hemoglobin (Hb) concentration in the blood fell to 3.5–7.5 g/dl (238 wounded and sick), they added separate plasma expanders or freshly frozen plasma (200 ml per 450 ml of Perftoran). In critical situations, Perftoran permits the delay, reduction (by two to three times) or avoidance of donor blood transfusions. Various side effects were found in about 8 per cent of patients; these included transient itching, hyperemia and dizziness, pain in the kidneys, and even hypotension and pulmonary complications (Maevsky *et al.*, 2001).

CURRENT USAGE OF PERFTORAN

Since its approval in 1997, Perftoran has been used for many clinical indications (Table 26.3). According to an All-Russian questionnaire (performed

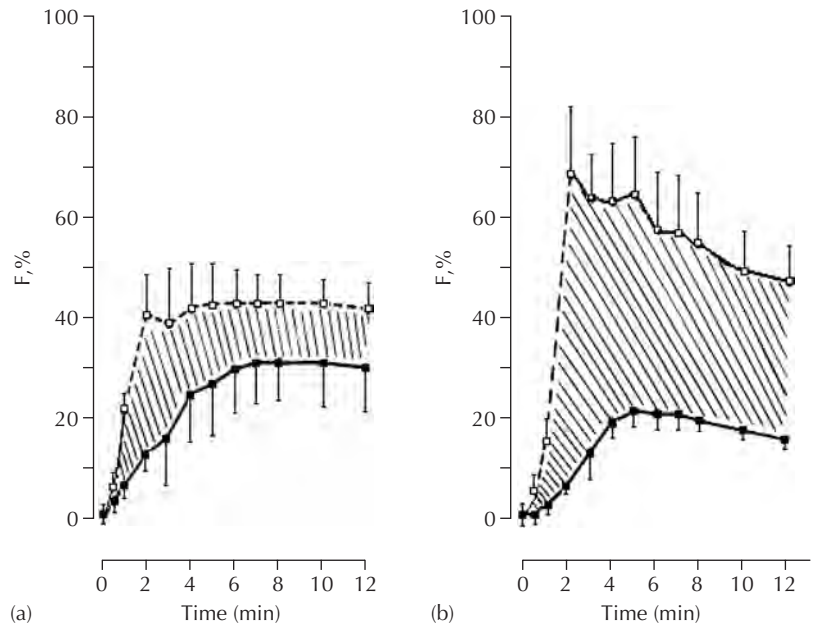

Figure 26.3 Reperfusion restoration of mechanical activity of rabbit heart with different perfusion medium after 40 minutes of total ischemia: (a) Tirode salt solution; (b) Perftoran supplemented with 2.5 mM calcium chloride. Continuous lines with dark marks show the level of diastolic tension; broken lines with open marks show the level of systolic tension; crosshatched regions show amplitudes of heart contractilities. F,% = isometric tension relative of the initial level.

Table 26.2 Patient distribution according to indications, doses of Perftoran and frequency of side effects during clinical trials (n = 964*)

Indications	Doses (ml/kg body weight)	Summary doses (l)	Patient distribution (%)	Frequency of side reactions (%)
1. Acute blood loss, hemorrhagic shock	6–30	1–5	22	2
2. Polytrauma, shock	4–12	0.4–1.2	20	0
3. Toxic shock	4–8	0.4–1.0	12.7	0
4. Limb ischemia	4–6	0.4–0.8	20.7	20
5. Cardiosurgery	–	1.0–2.0	11.1	0
6. Kidney transplantation	30	1.0–2.0	4.8	0
7. Burns, oncology and others	2–8	0.1–1.0	8.2	27
Total frequency of side effects				10**

*According to the original reports submitted in Russian Pharmaceutical Committee.
** Excluding cardioplegia and kidney transplantation patients.

by Evgeny Giburt at the Blood Center of the Russian Ministry of Health in 2002), positive effects of Perftoran were reported in 88.3 per cent of cases, negative effects in 3.3 per cent, the absence of any effect in 8.3 per cent, and side effects in 4 per cent of cases. The most frequent indication was bleeding (37 per cent), so the main Perftoran consumers were regional blood transfusion stations.

Perftoran was initially developed as a blood substitute to be used instead of allogeneic blood following massive blood losses, with simultaneous breathing of oxygen. In practice, Perftoran turned out to be useful even if the falling level of hematocrit and hemoglobin did not reach the transfusion trigger. As demonstrated by Tikanadze (1997), Perftoran administration (n = 32) in doses of 900 ml (100–120 drops/min) together

Table 26.3 The percentage of patients treated with Perftoran, by indication. Total number of patients = 3528, including 1921 Perftoran-treated patients in comparative studies (according to Russian Scientific literature 1997–2004)

Indications	Distribution of patients with Perftoran (%)
1. Blood losses, multiple organ dysfunction	37
2. Limb ischemia	14
3. Dysfunction of inflammatory response	8
4. Detoxication	4
5. Lung function damage	6
6. Cranial-cerebral trauma	7
7. Burns, thermal shock	3
8. Kidney transplantation	2
9. Cardiosurgery	3
10. Oncology	2
11. Local application: wound and ulcer healing, lavage of lungs, spinal cord and peritoneum	14

with crystalloids and colloids at $F_IO_2 = 0.4$ after the cessation of gastroduodenal bleeding of 1500–2500 ml provided the following: higher cardiac output due to an increase of the heart stroke volume to 60–68 ml (as opposed to 46–52 ml in the control group; $P < 0.05$); an increase in arterial and central venous blood pressure; and enhancement of arterial and venous PO_2, which were also more sustainable than those in the control group of 30 patients. According to Lasarenko *et al*. (2002), Perftoran was infused at the beginning of gastroduodenal and colon operations in doses of 400 ml (25 patients) or 800 ml (14 patients) after blood losses of 1000 or 2000 ml, respectively. Perftoran administration augmented microcirculation by 15–30 per cent ($P < 0.05$) in the liver, intestine, skeletal muscle and peritoneum; increased erythrocyte elasticity from 36 to 78 per cent of the normal level ($P < 0.05$); and decreased the blood viscosity from 87 to 54 cPs at 1/c ($P < 0.05$) when the normal level was 32 cPs at 1/c. In all these cases, Perftoran infusions were accompanied by acceleration of platelet aggregation and disaggregation, diminution of acidosis, and inhibition of peroxidative waste production in the blood by 1.5–2.0 times (Sofronov *et al*., 1999; Sofronov and Selivanov, 2003).

During lung resection with artificial ventilation ($F_IO_2 = 0.5$) when intraoperative blood losses were 400 ml (after hemodilution, hematocrit was 32–35), Perftoran administration (6 ml/kg) augmented saturation of HbO_2 in arterial blood to a significantly higher level than that in the control group treated with crystalloids (Biryukov and Petrova, 2001). As shown by Zakharov *et al*. (2001), inclusion of Perftoran in doses of 4–6 ml/kg in conventional transfusion therapy 1, 2 and 3 days after a hemorrhagic shock and additional operation bleeding increased the efficiency of reanimation treatment, and shortened the reanimation period and duration of artificial ventilation.

Usenko *et al*. (2002) gained much clinical experience in Perftoran application when treating bleeding, cranial-cerebral traumas, and burn shock. They adjusted Perftoran doses to the volume of blood losses (VBL) in the following ratios: 2–4 ml/kg for 20 per cent VBL, 4–7 ml/kg for 20–40 per cent VBL, 7–10 ml/kg for 42–70 VBL, and 10–15 ml/kg if VBL exceeded 70 per cent. On Perftoran administration they noted a decrease in arterial-venous shunting in the lungs, and an increase in PaO_2 and O_2 extraction from blood. As a result, sequelae dropped by 12.5 per cent and morbidity by 5–8.5 per cent. Usage of donor blood was reduced by 1.5 times. In most cases a positive effect was achieved after Perftoran application in doses of 4–6 ml/kg when the supplementary oxygen capacity of PFC emulsion was insignificant. These doses of Perftoran accelerated patients' resuscitation after cranial-cerebral traumas, and also the restoration of their mental activities. Side effects occurred in about 1 per cent of cases.

CLINICAL IMPLICATIONS

In compliance with the experimental data and the preregistration clinical trials, Perftoran efficacy was revealed not only as a blood replacement but also for treating polytrauma, different kinds of shock and brain injuries, and for the elimination of edema after cranial-cerebral traumas. It promotes rapid recovery from coma owing to fat or air embolism of the cerebral vessels, and prevents the development of the multiple organ dysfunction syndrome and the respiratory distress syndrome of adults (Sofronov *et al*., 1999; Usenko *et al*., 2002; Kligunenko *et al*., 2004). In cardiopulmonary bypass with hemodilution by Perftoran (45 patients) during reconstruction

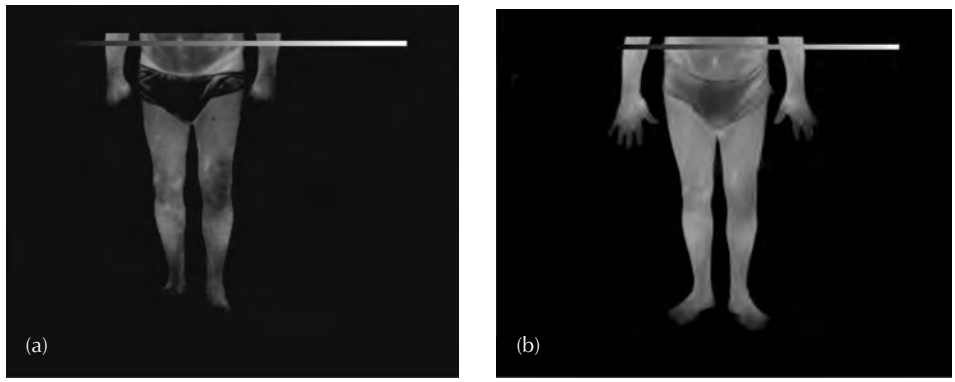

Figure 26.4 Thermovision pictures of ischemic limbs (a) before and (b) 2 hours after Perftoran infusion (200 ml), according to Ivanitsky *et al.*, 2003. (a) shows symptoms of 'limb amputation'.

operations on the heart, Kryuchenkov (1998) found a more pronounced antiacidosis effect (the lactate level decreased three- to five-fold), improvement of tissue oxygenation (O_2 delivery and extraction increased two-fold) and diminution of blood viscosity and erythrocyte injuries in comparison with control groups (60 patients, hemodilution with crystalloids or with mixture of crystalloids and banked erythrocytes). Moroz *et al*. (1995) found augmentation of skin PO_2 by 30 per cent after the infusion of 400 ml of Perftoran for limb ischemia, while dextran 40 augmented skin PO_2 by only 6 per cent. Thermovision images supported resuscitation of the blood flow immediately on Perftoran administration (Ivanitsky *et al.*, 2003; see Figure 26.4).

Repeated Perftoran infusion facilitates pain elimination at rest, and significantly enhances the distances for which patients can walk while remaining pain-free in 93 per cent of patients for about 6–9 months. Usenko *et al*. (2002) and Aliev *et al*. (2002) described the reduction of the ischemic area after acute myocardial infarct treated with small doses of Perftoran (100 ml). Infusion of 2000 ml of Perftoran into cadaver donors (no heartbeat) alleviated kidney transplant ischemic injuries. Reperfusion damage and rejection of kidney grafts also diminished after Perftoran infusion (4–6 ml/kg) into recipients (Onishenko *et al.*, 1990). Sofronov *et al*. (1997) demonstrated that Perftoran decreased the symptoms of poisoning with carbophos and neurotropic drugs. Stable antiinflammatory effects of Perftoran (1.5–3 ml/kg) were described by Moiseenko (1999) in chronic uveitis (39 patients). Combining Perftoran local lavage of injured spinal cord with intravenous administration, Katunyan *et al*. (2003) significantly improved the outcome of decompression operations and the neurologic resuscitation of patients after spinal cord trauma.

THE FUTURE OF PERFTORAN

We completely agree with Keipert (2003) that the most likely anticipated future PFC emulsion application (which has already been demonstrated by preclinical and first clinical efficacy data) will target tissue ischemia, with a focus on the vital organs. Since Perftoran enhancement is limited by the probability of side effects (about 4 per cent), which frequently result from violated conditions of storage, the possibility of decreasing the frequency of these side effects by technological modification and strict obedience regarding the rules of Perftoran usage will determine the imminent future of Perftoran application. It will lie in a wider scope of both previously mentioned and new indications, such as preoperative isovolemic blood replacement (during temporal autoblood harvesting), treatment of cerebral ischemia, reversal of myocardial ischemia, diminution of reperfusion injuries, resuscitation of emergency traumas, lung and peritoneal lavage, enhancement of sensitivity to radiation and chemotherapy, prolongation of the storage time of isolated organs prior to transplantation as well as detoxication, suppression of the hyperactive inflammatory response, and liver function correction.

SUMMARY

Perftoran can be qualified as an 'antihypoxic' and 'anti-ischemic' blood substitute with marked membranotropic effects. At present it is highly

advisable to use Perftoran at an early stage of blood replacement, when the joint functioning of Perftoran and the remaining erythrocytes can increase tissue oxygenation, delay and reduce allogenic blood usage, alleviate cerebral ischemic injuries, and prevent development of multiple organ dysfunction. Perftoran will target tissue ischemia and inflammation. Its further enhancement depends on the possibility of diminishing the frequency of side effects by technological modifications and stricter obedience regarding the rules of its usage.

EDITOR'S SUMMARY

Perftoran is a perfluorocarbon emulsion developed in Russia, with a similar composition to Fluosol. Perftoran is stabilized with Proxanol P268 instead of Pluronic F-68, both of which are copolymers, but the Russian studies found Proxanol P268 to be less toxic than Pluronic F-68 in animal tests. Perftoran contains no oncotic agents (starches), and therefore it can potentially be given in relatively large doses without concern of volume overload. Perftoran is bottled and frozen as a single solution – not three, as was the case for Fluosol. It is therefore more convenient to use.

Perftoran has a smaller particle size than Fluosol, to which the lower incidence of side effects such as anaphylaxis, flu-like symptoms and fever is attributed. In spite of its low O_2 capacity (6.9 ml/dl of O_2 at 760 mmHg PO_2), Perftoran was found to be efficacious in preclinical animal studies in a variety of species. Due to its low lipophilicity, its retention time in the body is quite long – 90 days in some studies. This was a concern, but no specific toxic effects have emerged from the studies.

Perftoran was approved for clinical use in Russia in 1997 after several trials involving 964 patients, some with massive blood replacement. The incidence of side effects is said to be 8 per cent. With increasing use in Russia, considerable clinical experience has now accumulated. Although it is being used in many different clinical indications, including acute and chronic anemia, its greatest application might be in oxygenation of specific tissues such as in limb ischemia, cerebral ischemia and coronary artery disease.

ACKNOWLEDGMENTS

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Chapter 27

Rational Development of Oxyfluor™

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INTRODUCTION

Oxygen is the currency of life. Oxygen deprivation of only minutes irreversibly degrades the function of cells, tissues and organs, causing death. Three of the four major killers in the western world (heart attack, stroke and hemorrhagic shock) are oxygen deprivation disorders. The integrated performance of the respiratory system, the cardiovascular system and the transport medium (blood) is dedicated to the 'critical' task of providing continuous delivery of oxygen to all the cells, tissues and organs of the body. However, oxygen transport is only one of many functions of blood. In recognition of this, modern transfusion practice provides specialized fractions of blood. Erythrocytes, the red blood cells, comprise the fraction specialized for oxygen transport. In recent years, there have been increasing shortages of whole blood and packed RBCs for oxygen transport. Even when whole blood or packed cells are available, there are many circumstances where they cannot effectively deliver oxygen to particular tissues in need. For example, following heart attack or stroke, RBCs cannot deliver O_2 to the infarcted areas.

Thus it is not surprising that there has been over 50 years of effort devoted to the development of new oxygen transport medium that can enhance oxygen transport or substitute for red blood cells where and when they are in short supply. These developments have centered largely on two approaches: purified hemoglobin (Hb) derivatives, and perfluorocarbon (PFC) emulsions.

This chapter is about the rational for development of HemaGen PFC oxygen transport product, Oxyfluor™. This chapter will try to explain why HemaGen is pursuing a PFC product rather than one based on Hb, and will summarize Oxyfluor's present state of regulatory development.

PFCs are extremely inert chemically and biochemically (Simon, 1947), have high gas solubility and diffusivity, and have extremely low toxicity. Unlike Hb and its chemically modified derivatives, PFCs do not enter into chemical reactions in the body and are excreted unmetabolized. Remarkably, PFCs can dissolve nearly 20 times as much oxygen as water, as well as the other respiratory gases, CO_2 and nitrogen. PFC oxygen content can be as high as 35–50 volumes per cent (Gjaldbaek and Hildebrand, 1949). In comparison, fully saturated Hb has an oxygen content of only 20 volumes per cent. Oxygen dissolves in PFCs in accordance with Henry's law and is not chemically bound, as it is with Hb. PFC-transported oxygen is therefore readily available by gradient diffusion and is delivered from plasma into tissue at twice the rate of Hb-transported oxygen. Breathing supplemental O_2 can increase O_2 off-loading from Hb in RBCs or

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Hb derivitives 1 volume per cent; O^2 off-loading from 40 per cent Oxyfluor can increase 13 volumes per cent. Changes in blood chemistry can substantially alter release of Hb-bound O_2; it does not alter release of O_2 dissolved in PFCs.

PFCs are synthetic molecules in which the hydrogens of hydrocarbons are replaced by fluorines. The fluorine–carbon chemical bond in PFCs is among the strongest covalent single bonds and makes the PFCs inert. PFCs were first synthesized in the 1930s in small quantities for research purposes. During World War II, the Manhattan Project developing the atom bomb developed the need for an especially inert solvent in order to separate the isotopes of uranium by diffusion of their hexafluroides. A crash program to develop suitable PFC solvents resulted in the post-war availability of a wide variety of structurally diverse and commercially useful PFCs.

EVOLUTION OF PERFLUOROCARBONS FOR OXYGEN TRANSPORT

Leland Clark

Leland Clark stimulated medical interest in PFCs with his dramatic demonstration that mice submerged in oxygenated liquid PFC could derive their entire physiologic oxygen requirements via the PFC in their lungs (Clark and Gollan, 1966). Neat PFCs must not be injected into the circulation, for they are immiscible in the blood and become emboli that can cause fatal vascular obstructions. Within a year of Clark's publication, Henry Sloviter reported that PFCs could be emulsified with serum albumin to be compatible with plasma upon intravenous injection (Sloviter and Kamimoto, 1967). Using this emulsion, he was able to extend the electrical activity of isolated rat brains much longer than with perfusion with rat RBCs. The following year, Robert Geyer reported on exchange transfusions of rat blood with an emulsion made of perfluorotributylamine, the surfactant Pluronic F-68 and physiologic salts. Animals survived on this PFC emulsion at high oxygen tensions until sufficient RBC regeneration enabled the animals to support life on room air (Geyer *et al.*, 1968, 1973). These rats developed normally and survived in apparent good health for their customary life expectancy. These total exchange experiments dramatically demonstrated both the efficacy and the safety of PFCs. The fact that rats survived with hematocrits as low as 3 per cent demonstrated the physiological gas transport capability of PFCs. The safety of the emulsions was evidenced by the replacement of serum albumin, immunoglobulins, clotting factors, platelets, leukocytes and erythrocytes, which had been removed by the near total exchange transfusion, indicating functional livers and bone marrows. Subsequently, clinical researchers initiated animal studies that identified many potential clinical applications for PFC oxygen transport agents, including therapy for hemorrhagic shock, heart attack and stroke, and potentiation of cancer therapy, etc.

Green Cross and Fluosol®

The entrance of the Green Cross Pharmaceutical Company of Japan into this field resulted in the preparation of a commercial PFC oxygen transport product, Fluosol-DA20 ('Fluosol'), the only synthetic oxygen transport product approved by the US FDA.

Fluosol-DA20 is a 20 per cent by weight, 10.6 per cent by volume emulsion of two PFCs, perfluorodecalin (PFD) and perfluorotripropylamine (PFTPA), emulsified by Pluronic-F68 containing a small amount of egg-yolk phospholipid (EYP). It is not stable in the liquid state and must be stored frozen (Mitsuno *et al.*, 1984; Yokoyama *et al.*, 1984). The required addition of PFTPA to the formulation for stability purposes detracts from the emulsion's medical usefulness because its half-life in the liver and other organs of the reticuloendothelial system (RES) is longer than desirable (65 days). Finally, the fact that the emulsion contains only about 10.6 per cent PFCs by volume limits its therapeutic efficacy because of low oxygen transport capacity. However, Fluosol-DA20 was a well-defined, reproducible product, and its availability led to generation of consistent physiologic and toxicological data. In addition to data from animals, Fluosol was administered to thousands of patients for several potential indications over several years. Many of these patients received Fluosol as part of a clinical trial, but many others received Fluosol because they refused blood transfusion for religious reasons. In a study of this latter group, with Hb levels less than 10 gm per cent, Fluosol failed to provide any more than a transient increase in arterial oxygen levels (Gould *et al.*, 1986). Fluosol did, however, contribute 28 per cent of the oxygen consumption of these patients. Because of the low PFC concentration in Fluosol, the gain in oxygen carried by

the PFCs was offset by the dilution of the hemoglobin and decrease in oxygen carried by the red cells. The conclusion of Gould's study was that Fluosol-DA20 was safe but ineffective.

The early clinical trials in Japan and the USA were at doses of 20–30 ml/kg of emulsion. Subsequently, dosages of emulsion went up to 40 ml/kg as a single dose and 56 ml/kg in repeated doses (2, 3, 4 and 5.6 ml of PFC). Despite these very large dosages (for example, 40 ml/kg provided 3 kg of emulsion and over 0.5 kg of PFC), patients showed only minor toxic symptoms. These symptoms were benign, reversible, and without meaningful functional impairment. In the various studies, from 10 to 30 per cent of patients experienced flu-like symptoms beginning 4 hours after infusion and generally disappearing at about 24 hours. These were found to be due to the surfactant Pluronic-68 (Tremper *et al.*, 1984), which causes C3 conversion and generation of C5a-related neutrophil-aggregating activity, transient neutropenia and thrombocytopenia. These adverse reactions were readily blocked by corticosteroids. In addition there was a temporary increase in the size of the liver and spleen, wherein the RES culled PFC particles from the bloodstream, and transient elevation of some liver enzymes (SGOT, SGPT and alkaline phosphatase).

These data strongly suggest that PFC emulsions can be administered safely in the therapeutic range. The FDA approved Fluosol-DA20 only for perfusion of arteries distal to obstructions during angioplasty procedures. With the introduction of catheters that perfused blood distal to the site of angioplasty, this market for Fluosol-DA20 eroded and Green Cross Pharmaceuticals withdrew the product.

HemaGen and Oxyfluor™

HemaGen Ltd was created in 1982. Shortly thereafter, HemaGen licensed Leland Clark's early technology and began supporting Dr Clark's research at the Children's Hospital Medical Center in Cincinnati. In 1986, HemaGen established a research laboratory to develop this technology further. In 1988, HemaGen sublicensed technology to Green Cross only for the Fluosol application (a formulation HemaGen had rejected) to enable it to enter the commercial market. In examining over 106 PFCs in emulsified form, HemaGen characterized PFC attributes and the relationships of structure to performance, leading to the synthesis of a novel emulsion composition containing what HemaGen believes to be the optimum PFC for oxygen transport purposes, perfluorodichlorooctane (PFDCO) (Kaufman *et al.*, 1998).

DEVELOPMENT OF OXYFLUOR™

The emulsification system

As demonstrated by the experience with Fluosol-DA20, in order for a PFC emulsion to be useful as an oxygen transport agent it must contain large amounts of PFC and also exhibit chemical and physical stability. This chemical and physical stability must persist during terminal sterilization at 121°C, under the desired condition of storage and during *in vivo* use. While much of the available literature focuses on the particle size stability of PFC emulsions (Krafft *et al.*, 1992; Pelura *et al.*, 1992; Riess *et al.*, 1992; Ni *et al.*, 1994; Postel *et al.*, 1994), few studies focus on the chemical stability of such emulsions (Song *et al.*, 1994). Although the PFC active ingredients are inert, the current emulsifier of choice, egg-yolk phospholipid (EYP), does not share that desirable characteristic. Since chemical degradation of EYP can lead to the production of toxic agents (Wretlind, 1981), it is necessary to assure stability of the emulsifier in order to ensure stability and safety of the end product.

Intravenous grade EYP is a complex natural product whose major constituents are phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE). The principal hydrolytic degradation products of these are lyso-PC (LPC) and lyso-PE (LPE), as shown in Figure 27.1.

Nearly 40 years ago, Arvid Wretlind solved the problem of emulsification of triglycerides for use in parenteral nutrition by using small amounts of purified EYP as surfactant (Wretlind, 1965, 1981). The resultant fat emulsions have been administered in very large quantities worldwide for three decades with great safety. These fat emulsions typically employ about 1.2 per cent (w/v) EYP as an emulsifier for 20–30 per cent triglyceride formulations. However, in using EYP to emulsify high concentrations of PFCs, substantially larger amounts of emulsifier are required. High concentrations of EYP in the emulsion can give rise to a significant problem with respect to the chemical stability of the emulsifier, which is affected by both oxidative and hydrolytic processes. The oxidative process can be easily controlled by the use of antioxidants and nitrogen blanketing of the product prior to heat sterilization and storage

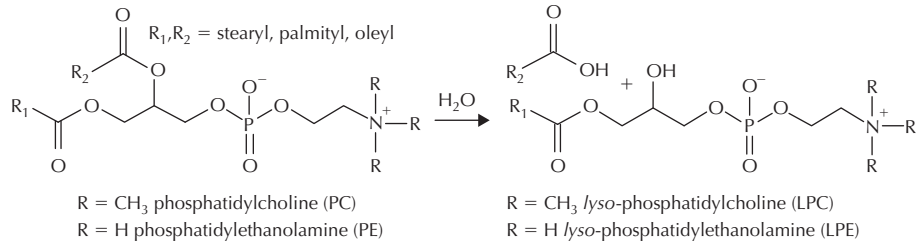

Figure 27.1 Hydrolysis of phosphatidyl choline.

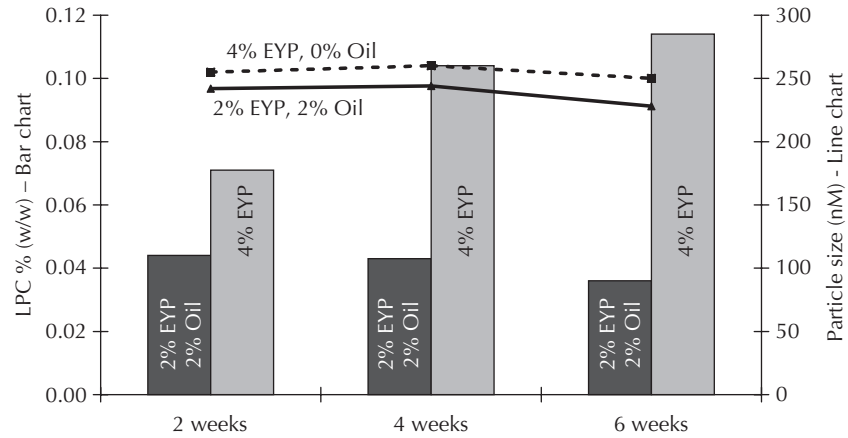

Figure 27.2 40°C storage – LPC and particle size.

(Long, 1991). However, the second problem, prevention of hydrolytic instability, is not so trivial.

HemaGen was keenly aware of the deficiencies of Fluosol that resulted from Green Cross's inability to provide a more stable emulsion: the concentration of PFC was too low; Fluosol could be stored only in the frozen state; and Fluosol stimulated the complement cascade. HemaGen was also aware that while the lipophilic hydrocarbon tails of the EYP surfactant interacted strongly with the triglyceride, they interacted poorly with the PFCs. HemaGen hypothesized that with the addition of triglyceride, the strong interaction between the EYP hydrocarbon tails and the triglyceride might cause the emulsion to restructure itself as a stronger emulsion than it was without the triglyceride. Experiments to test this hypothesis were conducted, and in all instances the addition of triglyceride strengthened the emulsions. HemaGen called this emulsification innovation the 'three liquid phase system' (3LP), in light of its three liquid components: water, PFC and triglyceride oil. While it was thought that the PFC might be covered by triglyceride, which may interface with the water, HemaGen did not know this to be the case and did not want the innovation to be so limited (Clark and Shaw, 1997). However the emulsion structured itself, it was empirically more stable than the control emulsions without triglyceride oil. The optimized PFC formulations required very low triglyceride levels – much less triglyceride than commercial fat emulsions. And, despite the large concentrations of PFC being emulsified (71.6 per cent w/v), it turned out that only very low EYP levels were needed to achieve a stable, heat-sterilizable product. Importantly, the aforementioned hydrolytic degradation of EYP was inhibited by this approach.

In Figure 27.2, the bar chart illustrates LPC production upon accelerated aging of perfluorooctylbromide (PFOB) emulsified with 4 per cent EYP contrasted with an identical PFOB emulsion containing 2 per cent EYP and 2 per cent oil. The PFOB emulsion with 2 per cent oil and 2 per cent EYP experienced far less hydrolysis than the PFOB emulsion with 4 per cent EYP. Thus, LPC levels in the presence of added oil do not change significantly over the course of the storage period. In contrast, the 4 per cent lecithin emulsion containing no added oil exhibited a drastic rise in LPC levels over time. The line segments

show the measured mean particle size as determined by quasi-elastic laser light scattering (QLS) during the course of the same study. Despite the lower lecithin content, the oil-containing emulsion demonstrates particle size stability equivalent to that observed for the high lecithin formulation. Both emulsions were heat sterilized under equivalent heat loads ($F_o \approx 15$). This striking result strongly suggests that the oil is contributing to the physical *and* chemical stability of the product.

HemaGen used this 3LP technique in emulsifying PFDCO in the later development of Oxyfluor. For this product stability studies of EYP only versus 3LP formulations extended to 18 months at both 4° and 25°C, and corroborated the exemplified PFOB accelerated aging study. The drug master file (DMF) for Oxyfluor specifies controlled *room temperature (25°C) storage for up to 12 months*. This represents extraordinary stability for a concentrated PFC emulsion, and negates the need for refrigeration of the product. The ability to use this product in the absence of refrigeration is of real importance, since substantial applications for this product are outside hospitals, where refrigeration is not practical. This feature allows the product to be stored and used in emergency vehicles.

The perfluorochemical

Having enhanced the emulsification process, HemaGen then turned to optimizing the perfluorocarbon. HemaGen evaluated over 106 PFCs from 1987 through 1996. The idea was to emulsify candidate PFCs, inject each of the emulsions into groups of 10 rats, and determine their profile with regard to a screen of four key biologic properties. Efforts were made to relate PFC structure to biologic properties, with an eye toward synthesis of novel structures to minimize toxicity and maximize efficacy.

The four biologic properties (see Table 27.1) for which the PFCs were screened were:

- high dose lethality
- hyperinflated noncollapsible lung syndrome (HNLS) measured as body-weight-normalized lung volume
- organomegaly measured as body-weight-normalized spleen weight
- tissue residence time, expressed as half-life of the PFC in the liver.

The tests were performed in sequence for each PFC. Table 27.2 presents the results for six PFCs of interest: Perfluorodecalin (PFD); Fluosol – a mixture of 70 per cent PFD and 30 per cent PFTPA; PFOB, one of the constituents of Oxygent™; and three novel compounds provided to HemaGen under four agreements with PFC manufacturers. One of these, perfluoro-1,8-dichlorooctane (PFDCO), was found to be particularly useful as an oxygen transport agent by HemaGen scientists and personnel of a PFC manufacturer, after extensive collaborative reviews of structure–activity data (Kaufman *et al.*, 1998).

HemaGen believed at the time of these studies (and assumed most investigators did) that, given a stable emulsification system, many PFCs would

Table 27.1 Preliminary rodent screen

Study	Dose, ml/kg PFC	Criterion
High dose lethality	16	100% survival
Lung volume @ 72 h	8	< 1.10 ml/100 g body weight
Spleen weight @ 72 h	8	< 1.20 g/100 g body weight
Liver $t_{1/2}$	4	< 10 days

Table 27.2 Results of preliminary rodent screen on selected PFCs

Treatment	Lung volume (ml/100 g)	Spleen wt (g/100 g)	$t_{1/2}$ (days)	CST (°C)	BP (°C)
PFD	**2.20**	**1.34**	3–4	22	142
70:30 PFD:PFTPA	**1.38**	0.96	4:65	22:44	129
PFOB	1.10	0.96	6–8	−25	141
PFDCO (*F*-1,8-dichlorooctane)	0.91	0.92	6–8	<−35	156
PFHBC (*F*-(4-hydrido-butoxy)-cyclohexane)	0.92	0.86	<1	17	146
PFTMCH (*F*-tetramethylcyclohexane)	0.76	1.09	**14–28**		146

Abnormal values are shown in bold type.

prove to be suitable candidates. In fact, nearly all of the compounds studied, including those discussed herein, passed the high dose lethality test.

Table 27.2 illustrates several points of interest. PFD is a poor choice, failing both the HNLS and the organomegaly tests. The addition of the higher molecular weight PFTPA to PFD to form Fluosol-DA ameliorated organomegaly but did not adequately ameliorate the HNLS test, which Fluosol failed.

Of greater interest is the relative performance of the PFCs most widely studied in the 1990s, PFOB and PFDCO. As shown in Table 27.2, a dose of 4 ml/kg results in the same clearance characteristics for both compounds. Based on the Yamanouchi study (Yamanouchi *et al.*, 1985), one would expect PFDCO to have a shorter tissue half-life than PFOB, since it is more lipophilic. In all hydrocarbons and bromoalkanes studied to date, the critical solution temperature (CST) of PFDCO is ~10°–12°C less than that of PFOB (Le *et al.*, 1996). However, in HemaGen's direct comparison studies there was never a statistically significant difference between tissue residence times for the two compounds. (Note that although Riess (2001) reported that PFDCO has a longer tissue residence time than PFOB, he did so by comparing a low dose (2.7 g/kg = 1.4 ml/kg) to the higher dose employed in HemaGen studies (4 ml/kg). Since the dose-dependent clearance of PFCs is well established (Yokoyama *et al.*, 1975; Mitten *et al.*, 1988; Yong *et al.*, 1996), such a comparison provides no information on the relative clearance rates of the two PFCs in question.)

In the HNLS Lung Volume Test, PFOB is at the margin between pass and fail, while PFDCO is solidly in the passing range. Both compounds pass the organomegaly test, with PFDCO garnering a somewhat better numerical score.

The liver half-life of the cyclic hydrido-ether, PFHBC, is astonishingly low (Moore *et al.*, 1996). No trace of this compound can be detected in the liver within 2 days of infusion, and there is no observable HNLS. These findings are surprising in light of its higher molecular weight, lower lipophilicity, and higher boiling point relative to PFOB. Riess and Tsuda suggest that increased lipophilicity is associated with shorter tissue residence time, given similar boiling points and molecular weights (Tsuda *et al.*, 1988; Riess, 2001). PFHBC is a compound of low lipophilicity and high boiling point, relative to PFOB, being eliminated at a much faster rate than either PFOB or PFDCO. HemaGen has not yet determined if the compound is subject to a different excretion pathway. However, a lower molecular weight alkyl hydride, (3-hydridopropyl-)-cyclohexane, provided similarly encouraging data in the rodent model only to prove lethal in higher animal studies.

In general, pure unsubstituted *F*-alkanes caused increased organomegaly, as shown by increased relative spleen weights for perfluorotetramethylcyclohexane (PFTMCH, $C_{10}F_{20}$) and PFD. Interestingly, an isomer of PFTMCH has successfully completed US FDA Phase I studies. This development should be followed with interest, as it illustrates a compromise between moderately unfavorable tissue residence time and organomegaly, and acceptable lung function. The listing of 14–28 days for liver $t_{1/2}$ of PFTMCH is due to the fact that at 4 ml/kg PFC, the clearance was bi-phasic and not amenable to first order analysis. HemaGen deemed such a lengthy clearance to be of concern, in light of the fact that PFC residence times in certain tissues such as bone marrow in rat are markedly increased relative to liver. In fact, Yokoyama *et al.* (1978) reported that even PFD is detectable in the marrow at 8 weeks post-infusion of a 4 g/kg dose.

The screening studies dictated PFDCO as the PFC of choice for the HemaGen formulation. However, because clinical testing of PFOB had already been reported, HemaGen decided to continue comparisons of PFDCO and PFOB in higher animals. Attention then turned to the baboon model to further distinguish between the two PFCs.

At a PFC dose of 8 ml/kg, baboons infused with PFOB exhibit marked increase in breathing rates that peak at 400 per cent of pretreatment values during the first week after treatment, and greater than 250 per cent of pretreatment values at 30 days following treatment. Although only the respiratory rate is shown in Figure 27.3, measurements of other pulmonary function tests (inspiratory capacity, vital capacity, functional residual capacity, residual volume and expiratory reserve volume) were all substantially altered. In addition, PaO_2 was substantially reduced in the PFOB treatment group. HemaGen observed this complex of findings of HNLS in both rodents and baboons. Flaim (1994) claims that HNLS is species-dependent, and has never been observed in humans. Is it possible that HNLS has not been observed in humans because of the relatively low doses of PFCs administered to humans to date? Published data from studies of Oxygent in human clinical trials have been limited to 2.7 g/kg.

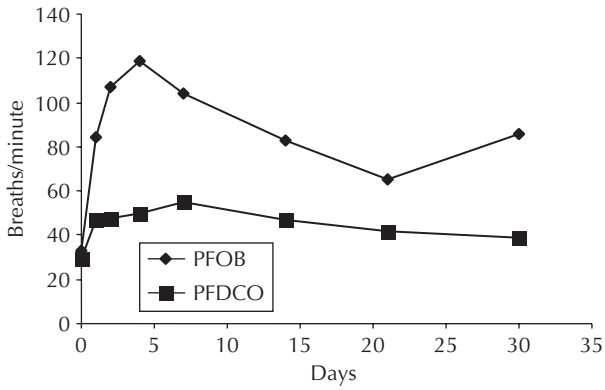

Figure 27.3 Testing in baboons: respiratory rate at 8 mL/kg of PFC.

This represents 1.4 ml/kg of PFOB – far less than the 8 ml/kg of PFOB in the aforementioned baboon study. Moreover, Oxygent, although comprised solely of PFOB in the early 1990s, is now comprised of a mixture of PFOB and perfluorodecylbromide (PFDB) (ratio PFOB:PFDB = 58:2). As discussed concerning the addition of PFTPA to PFD (see above), addition of a higher molecular weight substance has been seen to ameliorate (but not eliminate) the effects of HNLS. There may be certain risks associated with the use of PFDB. PFDB is a solid material at room and body temperatures (m.p. 49–51°C), and has a claimed tissue half-life of 23 days at 2.3 g/kg (Weers *et al.*, 1994) and up to 40 days at 6 g/kg (J. Weers, 2004, personal communication with T. Richard). Because PFDB has a much longer tissue half-life than PFOB (6–8 days), PFDB may remain in the body as a solid after PFOB has been eliminated.

As described previously, HemaGen sought to enhance the chemical stability of PFC emulsions utilizing EYP surfactants, by minimizing the amounts of EYP required and therefore reducing the amounts of their toxic hydrolytic end products. In contrast, Alliance Pharmaceutical ('Alliance') sought to enhance the physical (particle size) stability of Oxygent emulsions by focusing on the Lifshitz–Slesov equation, which characterizes the Ostwald ripening of PFC emulsions (Riess, 1998). This equation states that, when applied to PFC emulsions, the rate of increase in droplet volume is proportional to the interfacial tension, molar volume, solubility and diffusibility of the PFC in water. Accordingly, Alliance added a small amount of high-molecular weight and lipophilic PFDB to the PFOB of the Oxygent emulsion to decrease the solubility and diffusibility of the PFC mixture in water. It appears that in order to decrease the molar volume of the PFC in the Lifshitz–Slesov equation, Alliance also established a maximum of 2.7 g PFC/kg as the therapeutic dosage for Oxygent. In publishing studies of hundreds of clinical trial patients treated with Oxygent, the reported human dose of PFOB:PFDB based Oxygent is 1.8 g PFOB/kg (Hill *et al.*, 2002; Spahn *et al.*, 2002), occasionally followed by an additional 0.9 g/kg dose. This limitation, of course, also minimizes observable side effects, since the side effects are dose-dependent. However, it also places a limitation on the therapeutic value of the emulsion product, since the rate of oxygen delivery is also strictly proportional to the concentration of PFC. It should be pointed out, that in the Oxyfluor™ surgical patient clinical trials the highest concentration of PFC already administered is 3.58 g PFDCO/kg body weight in contrast to the published 2.7 g PFOB: PFDB/kg body weight of Oxygent®. In comparing product dosage, Oxyfluor™ is more concentrated than Oxygent® both by weight (71.6 per cent vs. 60 per cent) and by volume (40 per cent vs. 31 per cent).

HemaGen wishes to correct several misrepresentations regarding PFDCO in the current literature. Riess (2001) claims that PFDCO has a significantly lower O_2 solubility than PFOB, and that it has a significantly longer tissue residence time than PFOB. Neither of these statements is supported by currently available data. HemaGen has shown by experiment (see above) that there is not a tissue-residence time difference between the compounds, except that PFDB, one of the Oxygent constituents, is a solid that has a much longer tissue residence time than PFDCO. With respect to O_2 solubility, while it is true that PFOB dissolves 47 (vol.%) O_2 (Costa-Gomez *et al.*, 2004) as opposed to 43 (vol.%) O_2 for PFDCO, this does not imply increased solubility on a weight basis. The density of PFOB is 1.93 g/ml, while the density of PFDCO is 1.79 g/ml. Given equal weights of PFCs, both will dissolve similar amounts of oxygen, within the experimental error cited by Costa-Gomez *et al.* (2004). In the same article cited earlier, Riess (2001) states that PFDCO is prepared by electrochemical fluorination (ECF), thereby implying a diverse mixture of isomers in PFDCO. This statement is simply untrue. PFDCO is prepared by a direct fluorination procedure that does not give rise to the complex mixtures associated with ECF production.

OXYFLUOR REGULATORY STATUS

Limitations of space make it necessary to summarize, in table form, the studies that have been submitted to the US FDA. Commentary is added with regard to particularly important features.

HemaGen has completed the following studies on the path to regulatory approval:

1. A complete pharmacology, toxicology and pharmacokinetic package (see Tables 27.3)
2. Additional screens in rodents and primates
3. Successful efficacy studies of animal models of hemorrhagic shock, surgical hemodilution, and cerebral protection during cardiopulmonary bypass (see Table 27.4)
4. Phase I human clinical studies in healthy volunteers (see Table 27.5)
5. A Phase Ib randomized, single-center safety, tolerability and efficacy study of escalating doses of Oxyfluor™ administered intravenously to surgical patients pretreated with dexamethasone (see Table 27.5).

A Phase II randomized, single-center safety, tolerability and efficacy study of escalating doses of Oxyfluor administered during cardiopulmonary bypass of coronary artery bypass graft patients to reduce cerebral injury was initiated at the Hammersmith Hospital, London.

These clinical trials were progressing to the satisfaction of the Principal Investigator and

Table 27.3 Preclinical pharmacology, toxicology and pharmacokinetic studies

Study name	Species	Dose of PFDCO	Results
Oxygen solubility	NA	NA	• 17 vol % at the current concentration; solubility in neat PFDCO is ~43 vol%
Oxygen affinity of flood	Human whole blood	PFC:blood ratio 0:1, 1:8, 1:4, 1:2	• Hemoglobin dissociation curve is normal when mixed with Oxyfluor™
CV pharmacology	Dog	8 ml/kg	• Does not affect cardiovascular function in the dog
Microcirculation	Rat	4 ml/kg	• No effect in the microcirculation of the rat
Pentobarbital sleep time	Rat	1, 4 ml/kg	• Dose-dependent, reversible alteration in hepatic biotransformation of xenobiotics
Complement activation *in vitro*	Human plasma	PFC: Plasma 1:1, 1:4, 1:8, 1:16, 1:32	• No complement activation (Rosoff *et al.*, 1998)
Complement activation *in vivo*	Baboon	2 ml PFDCO	• Self-limiting increase in C3a • No activation of C5a • No activation of complement cascade
RES blockade Listeria	Rat	1, 4 ml/kg	• Transient blockade of the RES
RES blockade endotoxin	Rat	1, 4 ml/kg	• Transient blockade of the RES
RES blockade reversal *Staph. aureus*	Rat	4 ml/kg	• Alters host response to *S. aureus* infection, but is controlled by antibiotic therapy
RES blockade reversal *E. coli*	Rat	4 ml/kg	• Alters the host response to *E. coli* infection, but is controlled by antibiotic therapy
LD_{50} rangefinder	Rat	16, 20, 24, 28 ml/kg	• The LD_{50} is 21 ml PFDCO/kg in the rat
Acute toxicity – rats	Rat	1, 4, 8 ml/kg	• No effect @ 1 ml/kg • Effects @ 4 & 8 ml/kg, which return to normal at 61 days
Acute toxicity – baboons	Baboon	1, 4, 8 ml/kg	• Few effects @ 1 ml/kg • Effects @ 4 & 8 ml/kg, which return to normal at 60 days • No adverse pulmonary events
Hemolysis *in vitro*	Human blood	1:100, 3:100, 10:100 dilution of emulsion to blood	• No hemolysis of human blood
Mutagenicity	*Salmonella typhimurium*		• Not mutagenic
Pharmacokinetic/ ADME – rat	Rat	1, 4 ml/kg	• Clearance in 2 days @ 1 ml/kg • Clearance in 6 days @ 4 ml/kg

(*Continued*)

Table 27.3 *Continued*

Study name	Species	Dose of PFDCO	Results
ADME excretion/closure – rat	Rat	4 ml/kg	• Not metabolized • Excreted by exhalation or transpiration
ADME supplemental – rat	Rat	1, 4, 8 ml/kg	• Dose-dependent; clearance decreases with higher doses
Pharmacokinetic – baboon	Baboon	1, 4 ml/kg	• Dose-dependent; clearance decreases with higher doses
ADME supplemental – dose escalation	Baboon	1, 4, 8 ml/kg	• Few effects @ 1 ml/kg • Effects @ 4 & 8 ml/kg, reversible with time
ADME supplemental – intermediate dose	Baboon	2, 3 ml/kg	• Primates essentially unaffected
Platelet function	Monkey	4 ml/kg	• Transient decrease in platelet number resolved spontaneously, with no evidence of bleeding • No impact on platelet function
Dexamethasone pretreatment study in primates	Baboon	1, 2 ml/kg	• Pretreatment eliminates all side effects such as flu-like symptoms (Goodin and Kaufman, 1997)

Table 27.4 Animal model efficacy studies

Study	Species	Dose of PFDCO	Method	Results
Shock resuscitation (Goodin *et al.*, 1994)	Dog	2 ml/kg, 4 ml/kg	Two groups (n = 8) resuscitated with PFDCO or lactated Ringers	• Arterial oxygenation significantly improved in the PFDCO animals vs controls • PvO_2 restored to pre-shock levels only in PFDCO-treated dogs during first 60 min after fluid administration
Surgical hemodilution (Kaufman *et al.*, 1993)	Dog	1 ml/kg, 2 ml/kg, 4 ml/kg	Animals (n = 5) isovolumically hemodiluted and ventilated	• Pre- and post-PFDCO infusion hematocrit (20 vol%) and fluorocrit (5 vol%) remained stable • PFDCO caused significant ($P < 0.05$) increases in arterial and mixed venous oxygen tensions (PaO_2 & PvO_2), oxygen delivery and consumption versus breathing 100% oxygen alone • PFDCO and plasma phases maintained essentially all of the animals' oxygenation requirements • The contribution of red cells to tissue oxygenation was $\leqslant 12\%$ • PFDCO had little or no effect on hemodynamic and acid–base parameters
Cerebral protection during CPB (Cochran *et al.*, 1997)	Swine	4 ml/kg	Animals (n = 16) underwent CPB with crystalloid prime or an identical prime solution + PFDCO.	• The addition of Oxyfluor to the CPB prime significantly reduces the incidence and severity of the physiological and morphological effects of a massive air embolism introduced during bypass

(*Continued*)

Table 27.4 (*Continued*)

Study	Species	Dose of PFDCO	Method	Results
			Air insult delivered into the right carotid artery	• Almost all of the animals in the crystalloid-only group had extensive cerebral infarcts while no infarcts were present in any of the animals treated with Oxyfluor
Cerebral protection during CPB using retinal angiography to assess CNS protection (Herren *et al.*, 1998)	Swine	2 ml/kg	Animals (n = 20) underwent CPB with crystalloid prime or an identical prime solution + PFDCO. Air insult delivered into right carotid artery. CPB continued 1 hour following air embolism	• The group with crystalloid prime alone had more significant areas of capillary dropout, regions of non-perfusion and more vascular leakage than the Oxyfluor treated group • The addition of Oxyfluor to the CPB prime maintained the integrity of the blood-retinal (analogous to the blood-brain) endothelial barrier in the presence of a massive air embolism
Cerebral protection during CPB using retinal angiography to assess CNS protection (Taylor *et al.*, 1992)	Dog	1, 4 ml/kg	Animals (n = 10) submitted to 90 minutes of CPB (4 control, 3 @ 1 ml; 3 @ 4 ml)	• Oxyfluor reduces incidence of microembolism during CPB as measured by retinal angiography • The control group had high incidence of retinal occlusions • The low-dose group had reduced incidence of retinal occlusion • The high-dose group had no retinal occlusion

HemaGen. Partly through the first cohort of 20 patients receiving the lowest dosage of Oxyfluor, HemaGen's strategic partner unexpectedly withdrew funding from the clinical trials. At the time this action was taken, there had been no adverse event attributable to Oxyfluor; nor was there any suggestion of a clinical hold by the regulatory agency involved.

Ten years earlier, during the invention and development of Oxyfluor, HemaGen had entered into a joint venture partnership with a 'Big Pharma' corporation. Under the series of agreements entered into, HemaGen's responsibilities to that partnership involved the development of Oxyfluor and funding that development to the point of successful submission of an Investigational New Drug Application (IND) allowing clinical trials to be initiated. Under the terms of the partnership agreements, the strategic partner's duty was to fund the clinical trials and other regulatory costs subsequent to the successful IND filing, so long as Oxyfluor was in clinical trials. The agreements went on to provide that if and when Oxyfluor received regulatory approval, the strategic partner would market Oxyfluor for agreed-upon indications in agreed-upon geographies.

In tandem with the Oxyfluor PFC program, the strategic partner independently pursued an Hb-based oxygen transport program. This internal program had advanced to multi-center clinical trials in the USA and Europe. A costly manufacturing facility for production of the Hb-based product had been built in Europe. Shortly before the strategic partner terminated funding for the HemaGen program, the US FDA and the EMEA terminated the four pivotal clinical trials of the Hb-derived product in the USA and Europe because of excess mortality in the treatment groups. The strategic partner's response to this regulatory development was to terminate all of its oxygen transport programs, including HemaGen's. Since this action was taken unilaterally and summarily, funds were not available to continue the clinical trials. HemaGen intends to resume these promising clinical trials when suitable alternative funding is in hand.

Table 27.5 Clinical trials

Study	Objective	Status	Results
Phase Ia Safety trial: healthy volunteers	Assess safety and tolerability of escalating doses (0.25, 0.50 and 1.00 ml PFDCO/kg) in normal healthy male volunteers	COMPLETE	• Subjects (n = 6) in the low-dose group were unaffected by treatment • Subjects (n = 6) in the mid-dose group had flu-like symptoms • All subjects (n = 5) in the high-dose group had flu-like symptoms and spurious decrease in platelet count
Phase Ib Safety trial: surgical patients	Assess safety and tolerability of escalating doses (1.0, 1.50 and 2.00 ml PFDCO/kg) in surgical patients pretreated with dexamethasone	COMPLETE	• Patients were generally unaffected by treatment • Pretreatment with dexamethasone greatly reduced flu-like symptoms • Side effects of PFDCO treatment, including decreased platelet count, were transient and dose-dependent
Phase I/II (US): cardiac surgery cardiopulmonary bypass	Assess safety, tolerability and efficacy of two doses (0.50 and 1.0 ml PFDCO/kg) in cardiopulmonary bypass/coronary artery bypass graft patients	VOLUNTARY TERMINATION BY HEMAGEN	• Principal investigator's institution was unable to provide adequate flow of suitable subjects. An administrative decision to transfer low-risk cardiac surgery to a neighbor institution was made shortly after onset of study
Phase I/II (European): cardiac surgery cardiopulmonary bypass	Assess safety, tolerability and efficacy of two initial doses (0.50 and in 1.0 ml PFDCO/kg) cardiopulmonary bypass/ coronary artery bypass graft patients	VOLUNTARY PAUSE BY HEMAGEN	• Program proceeding to satisfaction of PI and HemaGen • No product-related adverse events. • Funding for clinical trials terminated by Big Pharma strategic partner • PI wished to proceed with dose escalation at time of funding cessation

Table 27.3 adequately summarizes the pharmacology, toxicology and pharmacokinetic studies.

THE FUTURE OF OXYFLUOR AND HEMAGEN

The continuous delivery of a sufficiency of oxygen is critical to sustaining function and survival of every tissue and organ of the body. Accordingly, the development of an acellular oxygen transport medium that is instantly available for administration, usable without matching to each recipient, storable for long periods without refrigeration, provides superior oxygen delivery, free of the risk of infection and with a wide margin of safety, will find utility for a broad range of clinical applications in conditions and situations where fresh, grouped, typed and cross-matched blood is unavailable or is unable to provide adequate oxygen delivery. An example of the former is the trauma patient with bleeding and shock who requires transport to a distant hospital during the 'golden hour' for therapy, and could benefit from relief of hypoxemia during transport to a medical facility. An example of the latter is the patient with coronary occlusion and impending myocardial infarction whose red blood cells cannot transit beyond the arterial obstruction, but for whom oxygen-loaded, nanometer-sized PFC droplets or Hb-derived polymers may flow through the very small-caliber arterioles of the coronary collateral circulation to oxygenate the myocardium.

HemaGen has elected to pursue the PFC option because of the extreme biochemical inertness of PFCs, demonstrated by the excellent safety profile of Fluosol, a first-generation PFC formulation administered to several thousands of patients over several years. To HemaGen's thinking, this contrasts with what appears to be very much greater biochemical reactivity of Hb-based oxygen transport products. PFCs are not subject to oxidation, do not participate in free-radical

reactions, cannot scavenge nitric oxide, and are not metabolized and subject to metabolite-based toxicities – all dangers that Hb-based agents must try to avoid. PFC emulsions can be prepared to tight specifications from readily available materials by simple, well-understood processes that are easily validated and have withstood the test of decades in the safe, large-scale preparation of nutritional fat emulsions. In contrast, the raw materials of Hb-derived products are much more varied (outdated blood donations, animal blood, recombinant products), the processes may be more difficult to control and the end product more complex, although even small changes may have severe consequences. (In erythrocytes, for example, a change in only a single amino acid can result in cell sickling.) PFC emulsions will also have the cost advantage over Hb-based preparations.

Some may be discouraged by the several decades of oxygen transport development with the regulatory approval of only a single oxygen transport product, Fluosol – which lacked both adequate stability and sufficient PFC concentration to be widely efficacious. HemaGen has tried to keep its eye on the dimension of the problem and the importance of clinical success. Rather than selecting an obvious PFC and then trying to overcome its shortcomings by adding other PFCs, HemaGen has patiently undertaken a 9-year evaluation of over 106 PFCs to determine (and in the key instances create) an optimal PFC for wide medical application. Oxyfluor emulsion is made of an optimal PFC, perfluoro-1,8-dichlorooctane (PFDCO). It was designed and synthesized in collaboration with a PFC manufacturer. It is proprietary to HemaGen.

Of the four PFC emulsions covered in this volume, PFDCO is the only emulsion made of a single PFC. Utilizing a proprietary formulation technology (3LP emulsification), Oxyfluor is the only PFC emulsion with sufficient stability to be stored without refrigeration for a year – which is important for uses outside of hospitals, such as in ambulances, etc. To round out another desiderata of PFC oxygen transport agents (indicative of their therapeutic strength), Oxyfluor has been administered to humans at the highest reported PFC dosages, and is still in the dose escalation phase of its clinical trials.

HemaGen's clinical development program was interrupted as collateral damage to the unfortunate demise of a very large and longstanding Hb-derived oxygen transport program. However, it has become clear that the competition to provide an oxygen transport biopharmaceutical is a marathon rather than a sprint. HemaGen has not been substantially disadvantaged by this hiatus in clinical trials, since during this time events have transpired that have served to clarify and shake out the field. Nothing in these developments has changed the fact that PFDCO is the best PFC yet developed for use in acellular oxygen transport products. HemaGen sees a clear path to regulatory approval and is keen to reach that goal.

EDITOR'S SUMMARY

Oxyfluor(RM) is one of several 'second-generation' perfluorocarbon emulsions to be summarized in this volume. Like Oxygent™ (Alliance Pharmaceutical), Oxyfluor has a significantly higher oxygen capacity than Fluosol-DA. Oxyfluor was developed based on early work by one of the seminal workers in the field, Leland Clark, and the continued refinement of the formulation by scientists at HemaGen/PFC. The latter evaluated many PFC components and formulations, using rational criteria including tissue retention, the propensity to cause the unique 'lung hyperinflation' syndrome in animals, as well as room temperature stability. The PFC selected was perfluorodichlorooctane (PFDCO), which differs somewhat from perfluorooctylbromide (PFOB) used in the formulation of Oxygent. Phase I, Ib and II clinical trials with Oxyfluor were apparently successful, in that no safety concerns were raised, aside from mild thrombocytopenia and occasional flu-like symptoms. Further development and dose escalation of Oxyfluor is currently on hold pending financing of future trials. HemaGen/PFC is a small company that had formed a partnership with Baxter International to finance its clinical trials. When Baxter suffered major setbacks with its program based on hemoglobin-based products, they elected to abandon all work on therapeutic oxygen carriers, including its joint development with HemaGen/PFC of Oxyfluor.

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Chapter 28

Oxygent™, a Perfluorochemical-Based Oxygen Therapeutic for Surgical Patients

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INTRODUCTION

For many decades, scientists have actively pursued development of 'artificial blood' – seeking a product that is safe, universally compatible with all blood types, and readily available. Despite the safety of allogeneic (donor) blood having improved significantly in recent years, the public's perception of the dangers still associated with allogeneic blood remains high, fueled primarily by documented cases of viral disease transmission, and fatal hemolytic transfusion reactions due to clerical errors. Furthermore, new viruses and prions continue to be discovered and publicized in the media, and any of these could end up in the blood supply, where they might become a new transfusion risk in the future.

Oxygen therapeutics (typically referred to as 'blood substitutes') are anticipated to play an important role in easing the increasingly frequent shortages of donor blood (Riess, 2001). These agents would also avoid several transfusion-related safety issues, and could thereby profoundly change the practice of transfusion medicine in the future. Significant clinical development challenges remain, however, since regulatory approval of these products for a transfusion avoidance indication may require that they be proven to be as safe as allogeneic blood.

EMULSION CHARACTERISTICS

Over the past 15 years, Alliance Pharmaceutical Corp. (San Diego, CA) has been developing Oxygent™, a concentrated and stabilized second-generation perfluorochemical (PFC) emulsion. The current formulation contains 60 g PFC/dL (~31 per cent v/v) comprised of two active pharmaceutical ingredients; perflubron (perfluorooctyl bromide, $C_8F_{17}Br$) as the principal PFC and a small quantity of perflubrodec (perfluorodecyl bromide; $C_{10}F_{21}Br$) to stabilize particle size growth during storage (Riess, 1991). To make Oxygent, the PFCs undergo emulsification using pharmaceutical-grade egg-yolk phospholipid (the same surfactant typically used to emulsify other commercial lipid-based emulsion products – such as Intralipid™) in a buffered electrolyte solution. Both PFCs used in Oxygent have been produced in large-scale (metric ton) quantities and are available at pharmaceutical-grade purity (>99.99 per cent) from commercial suppliers. The oxygen solubility for the neat PFC used in Oxygent is ~53 ml/dl per atmosphere, resulting in a net oxygen transport carrying capacity of ~16 ml/dl per atmosphere for the 60 per cent w/v Oxygent emulsion formulation.

The manufacturing procedures and formulation for Oxygent developed and patented by

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Alliance comprise a simple, efficient, high-yield process that can produce emulsion particles with an average median diameter of 0.16–0.18 μm (Chapman *et al.*, 1996), approximately one-fortieth the size of a red blood cell. Oxygent is formulated in phosphate-buffered saline with an osmolality of 300–310 mOsm/kg, is buffered to neutral pH (7.0–7.2), and has a viscosity of ~4 cPs (25°C, shear of 1/s). Oxygent undergoes terminal heat-sterilization (temperature > 120°C) and is formulated as a ready-to-inject emulsion in 110-ml single-use glass vials sealed with a spikable rubber stopper and aluminum over seal. Oxygent has a 24-month shelf life when stored at normal refrigeration (2–8°C), but the emulsion is stable enough to tolerate exposure at room temperature (25–30°C) for up to several weeks.

NON-CLINICAL SAFETY

The safety of Oxygent has been evaluated in over 250 preclinical studies in multiple animal species. Extensive toxicology studies have demonstrated that Oxygent is well tolerated with no serious adverse effects at clinically relevant doses (approximately 1.0–6.0 ml/kg). These results were confirmed in several special safety-related toxicology studies performed to assess many different biological parameters, including systemic hemodynamics, blood components with emphasis on hemostasis and platelet function, immune function and host resistance, and pulmonary function. In addition, a series of ancillary pharmacology studies has demonstrated no significant changes in behavioral or physiological evaluations, locomotor activity, gastrointestinal motility, cardiovascular or respiratory evaluations, neuromuscular function, or interference with the efficacy of various common anesthetic and analgesic drugs.

The most common biological effects observed following infusion of PFC emulsions in preclinical studies include (1) a short-lived febrile response starting several hours after dosing, and (2) a transient drop in platelet counts at 2–3 days post-dosing, but with no adverse effect on hemostasis (i.e., platelet function and bleeding time remain normal). The mechanisms for both of these effects have been elucidated, and are related to the normal clearance of the emulsion particles from the circulation by phagocytic cells (liver Kupffer cells and splenic macrophages) of the RES and to the physical properties of the emulsion, particularly particle size and the choice of surfactant (Flaim, 1994). The clearance of Oxygent particles from the circulation is dose-dependent, and yields a blood half-life of approximately 12 hours, with most of the drug being cleared from the blood within 48 hours (Klein *et al.*, 1994). Once cleared from the blood by macrophages, the surfactant is degraded, leaving behind the pure PFC. Since PFC molecules are completely inert they are not broken down in the body, reducing any potential for toxicity from metabolic degradation products. Instead, intact PFC molecules simply diffuse slowly from tissues back into blood, where they are transported, dissolved in blood lipids, and then eliminated from the body over time via expired air (Riess, 2001).

The rate of PFC elimination from tissues depends primarily on vapor pressure and lipid solubility of the PFC (Weers, 1993), and the time required to clear PFC from the tissues is essentially dose-dependent. Second-generation PFC emulsions based on perflubron use PFCs that are more lipid-soluble than the original PFCs (e.g. perfluorodecalin) used in earlier first-generation dilute PFC emulsions such as Fluosol® and Perftoran®. Both the incidence and the magnitude of biological effects stemming from the activity of macrophages and phagocytic RES cells are related to the emulsion formulation, especially with respect to the emulsion particle size – i.e., smaller-diameter (0.1–0.2 μm) particles tend to be less detectable by the RES and thereby attenuate the biological effects (Keipert *et al.*, 1994).

Complement activation was occasionally observed after administration of Fluosol due to its synthetic Pluronic® surfactant (Ingram *et al.*, 1993), but this has *not* been observed with second-generation PFC emulsions like Oxygent that use egg-yolk lecithin as the only surfactant. Following administration of clinically relevant doses of Oxygent, there are no adverse hemodynamic effects (i.e., no vasoactivity) and no decreases in cardiac output, thereby allowing the PFC-dissolved oxygen to be effectively delivered to the tissues (Johnson *et al.*, 1995). In contrast to blood or blood-derived products, there is also no risk of person-to-person disease transmission when using Oxygent, since it is free of any blood-derived components.

CLINICAL SAFETY

To date, almost 1500 subjects have been enrolled in 20 clinical studies with Oxygent, including

Phase I healthy volunteer studies, and Phase II and III studies in surgical patients. Of these, more than 800 subjects have received Oxygent. The safety profile of Oxygent has been evaluated in several clinical studies involving healthy volunteers (four studies), cancer patients (six studies), general surgery patients (six studies), and cardiac surgery patients (four studies). Clinical studies with Oxygent in surgical patients have tested doses ranging from approximately 1.5 ml/kg (0.9 g PFC/kg body weight) up to 6.0 ml/kg (3.6 g PFC/kg, which represents about four 110-ml units of Oxygent for an average 70-kg individual).

The overall safety of Oxygent was investigated in detail in Phase I studies with healthy volunteers. As reported by Leese *et al*. (2000), a transient increase in body temperature was observed starting several hours after dosing in ~15 per cent of the treated awake subjects, but resolved within 12–24 hours. A mild transient decrease in total platelet count (<20 per cent drop from starting levels) was observed at 3 days post-dosing, which recovered to baseline levels by 7 days. Despite this delayed effect on platelet count, this study and the one reported by Noveck *et al*. (2000) also demonstrated the absence of any direct effect of Oxygent on platelet function (assessed by *ex vivo* platelet aggregation in response to agonists like arachidonic acid, collagen and ADP), and no prolongation of measured bleeding times or adverse impact on coagulation parameters (Leese *et al*., 2000). In addition, there was no evidence of 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 effects on blood chemistry; and no hemodynamic effects or signs of vasoconstriction – i.e., no blood pressure or heart rate changes (Noveck *et al*., 2000).

PRECLINICAL EFFICACY

The method of oxygen transport by PFCs is different from hemoglobin. Under normal conditions, about 20–30 per cent of the oxygen bound to Hb inside red cells is unloaded systemically and consumed by tissues. In contrast, Oxygent transports oxygen dissolved in the PFC droplets in proportion to the partial pressure of oxygen in the blood (PO_2), which can be substantially increased as the fraction of inspired oxygen (F_IO_2) that the patient is breathing is elevated. Hence, extraction of dissolved oxygen from a PFC emulsion is linear, and can exceed 90 per cent assuming an arterial PO_2 level of 500 mmHg (Keipert, 1998). As the Oxygent emulsion perfuses the microcirculation, dissolved oxygen is released initially from the PFC and the plasma, leaving a greater reserve of oxygen bound to the Hb inside the red blood cells; this makes Oxygent an attractive drug for a variety of clinical applications in which tissues may be at risk of acute hypoxia due to ischemia or transient anemia. This intrinsic property enables Oxygent to enhance tissue oxygenation even when administered in relatively small doses. Numerous preclinical studies have demonstrated that: (1) Oxygent supports tissue oxygenation; (2) the oxygen delivered by Oxygent is available to support metabolic processes at the tissue level; and (3) this improved oxygenation status translates into improved organ function (Flaim, 1998).

POTENTIAL CLINICAL APPLICATIONS

Tissue oxygenation and hemodilution

In normal animals under hyperoxic conditions, dosing with Oxygent was associated with a significant improvement in oxygen delivery and oxygen consumption in a dog model of maximally working isolated skeletal muscle (Hogan *et al*., 1992), and with an increase in plasma oxygen solubility and tissue PO_2 in both dog skeletal muscle (Gayeski *et al*., 1994) and cat retina (Braun *et al*., 1992) even when using low doses of Oxygent (e.g. 0.9 g PFC/kg). Oxygent has been shown in various studies to deliver oxygen to support metabolic processes, which correlate with improved organ function in critical tissues, including brain and heart. In the brain of normal awake rabbits (van Rossem *et al*., 1997) and anesthetized cats (Padnick *et al*., 1999), Oxygent dosing markedly enhanced cerebral cortical PO_2 above levels achieved with oxygen-breathing alone. In a model of partial brain stem ischemia in dogs using transient basilar artery occlusion following dosing with Oxygent, Guo *et al*. (1995) demonstrated a significant improvement in cerebral metabolic status and recovery of brain function (assessed by auditory evoked potentials) in the Oxygent-treated group.

Several studies have been performed using canine hemodilution models designed to mimic acute surgical anemia and blood loss (Flaim,

1998). These studies employed the use of a number of oxygen electrodes to monitor PO_2 levels in heart, brain, muscle, gut, and liver tissue to assess the impact of Oxygent-induced blood PO_2 increases on tissue oxygenation. Results clearly showed that improvements in tissue oxygenation with Oxygent treatment correlated with the observed increases in blood oxygenation (Keipert, 1998).

Studies were conducted in splenectomized dogs that were hemodiluted to a Hb concentration of 7–8 g/dl, administered Oxygent (doses from 0.9–5.4 g PFC/kg), and then subjected to volume-compensated blood loss to mimic intraoperative bleeding with fluid replacement (Batra *et al.*, 1997; Habler *et al.*, 1998a). In each of these studies, overall oxygenation status was significantly improved during blood loss in animals given Oxygent compared to controls, while breathing either room air or 100% oxygen. Throughout the bleeding phase, Oxygent maintained adequate systemic oxygenation at lower Hb concentration levels than was possible without Oxygent, and preserved local tissue oxygenation as assessed by PO_2 electrode measurements in the gut (Keipert *et al.*, 1996a) and in skeletal muscle and liver (Batra *et al.*, 1997). In particular, there was again a positive correlation between tissue PO_2 and mixed venous oxygen tension ($P\bar{v}O_2$) in this study (Keipert *et al.*, 1996b). As reported by Habler *et al.* (1998b), even after considerable hemorrhage, one dose of Oxygent (1.8 g PFC/kg) was as effective as about 3.8 g/kg of hemoglobin given as autologous blood in preserving adequate tissue PO_2 and maintaining better myocardial function as evidenced by improved left ventricular contractility. In addition, these studies demonstrated no untoward hemodynamic effects associated with the administration of Oxygent. Collectively, these non-clinical efficacy studies in surgical hemodilution models provided compelling evidence that Oxygent can prevent tissue hypoxia, and can preserve myocardial and cerebral function in the presence of acute anemia or ischemia.

Cardiopulmonary bypass

Studies have also been performed using canine models of cardiopulmonary bypass (CPB) to assess systemic and myocardial oxygenation parameters. Oxygent treatment resulted in a significant increase in mixed venous blood PO_2 ($P\bar{v}O_2$) levels (an indirect reflection of tissue oxygenation) versus controls in the absence of any changes in total oxygen consumption or hemodynamics (del Balzo *et al.*, 1996). Treatment was also associated with improved myocardial recovery post-bypass, as well as increased delivery and extraction of dissolved oxygen. In a study by Holman *et al.* (1995), in anemic (hemodiluted) dogs put on bypass, the additional dissolved oxygen provided by Oxygent appeared to be responsible for ameliorating post-CPB cardiac function after weaning from bypass and resulted in a lower mortality rate in the Oxygent-treated group.

During CPB, gaseous micro-emboli can be created from multiple sources (cannulation, venting of the heart, oxygenator) and may be partly responsible for the neurological and neuropsychological deficits often observed in patients after cardiac surgery (Spiess *et al.*, 1986). Pretreatment with Oxygent has provided benefits in terms of survival and recovery from transient neurological deficits in animal models. This protective effect may be due to a combination of the PFC's ability to resorb gaseous air micro-emboli (nitrogen, the main constituent of air, is ~25 times more soluble in PFC than in water at 37°C) and to deliver oxygen and improve perfusion of ischemic regions in the brain. A canine model of transient brainstem ischemia was able to demonstrate full functional recovery of auditory evoked potentials in Oxygent-treated dogs compared to saline controls (Guo *et al.*, 1995). These data collectively suggest that it may be possible in the future to use Oxygent to prevent tissue injury arising from gaseous emboli in the blood during cardiac surgery, and potentially to ameliorate cerebral injury from ischemic hypoxia during neurosurgery (e.g. aneurysm clipping).

Shock and trauma

A number of preclinical studies have demonstrated the potential for future applications of Oxygent in a variety of critical care settings. In a study of endotoxin-induced shock in a canine model, Cain *et al.* (1994) showed that Oxygent treatment increased oxygen uptake in the gut and in muscle, and improved regional blood flow and cardiac output. Using a model of hypotensive resuscitation from severe uncontrolled hemorrhage in swine breathing 33% to 67% oxygen, Stern *et al.* (1995) demonstrated enhanced oxygen delivery and improved survival with Oxygent. In another dog model of cardiac arrest, aortic arch perfusion with Oxygent was shown to

result in improved coronary perfusion and faster return of spontaneous circulation (Manning *et al.*, 1997).

Resuscitation of hemorrhagic shock in two rat models with Oxygent has demonstrated an improvement in cerebral oxygenation (Waschke *et al.*, 1994) and better restoration of hepatic energy metabolism (Paxian *et al.*, 2003). A recent study by Daugherty *et al.* (2004) analyzed the efficacy of using Oxygent in a rodent model of traumatic brain injury (TBI) using lateral fluid percussion. Results demonstrated that Oxygent treatment significantly increased cerebral oxygenation after TBI and enhanced mitochondrial function at 4 hours after injury as compared with saline controls.

Decompression sickness

Because of their high solubility for all gases, including nitrogen in air, PFCs have been proposed as a potential treatment for decompression sickness (Spiess *et al.*, 1988). Recently, using a swine model of severe decompression sickness (DCS) and post-dive treatment with Oxygent, Dromsky *et al.* (2004) were able to demonstrate that Oxygent-treated animals sustained significantly less DCS than the controls (53 per cent vs 93 per cent), and no animals in the Oxygent group sustained neurological DCS, which was present in 69 per cent of the swine in the other two groups. These preliminary findings suggest a potential future indication for using PFC emulsions to treat DCS when hyperbaric treatment is delayed or unavailable.

Organ preservation

Oxygent has undergone preclinical evaluation for preserving tissues and prolonging the storage time of an organ (e.g. kidney) prior to transplantation. Studies by Brasile *et al.* (1994) have demonstrated the ability to preserve canine kidney autografts without the need for extreme hypothermia by pulsatile perfusion using Oxygent-supplemented media at 32°C. Studies using isolated Langendorff rabbit heart preparations by Symons *et al.* (1999) have demonstrated that Oxygent supplementation of the perfusion media resulted in better maintenance of oxygen delivery, increased tissue oxygenation and high-energy phosphates, and improved myocardial function following low flow ischemia.

Tumor oxygenation

Another application that has been studied extensively in different preclinical animal models bearing various implanted tumors involves the use of PFC emulsions for augmenting PO_2 levels in hypoxic tumors to enhance the tumor's sensitivity to radiation and chemotherapy (Rockwell *et al.*, 1992; Teicher *et al.*, 1992, 1994). Many of these preclinical studies demonstrated the basic efficacy of this approach, and this eventually led to preliminary clinical studies with Fluosol in oncology patients (Evans *et al.*, 1993). To date, however, no company has chosen to pursue this indication as their first commercial application for a PFC- or Hb-based oxygen therapeutic, perhaps because of the challenging regulatory requirement to demonstrate improved mortality in order to gain approval for such an application in treating cancer patients.

Sickle cell disease

Finally, the vaso-occlusive crises that commonly occur in sickle-cell disease patients may present another future application where the immediate oxygenation benefit provided by Oxygent and other oxygen therapeutics might eventually prove beneficial (Rheindorf *et al.*, 1985). Recently, Kaul *et al.* (2001) has demonstrated the efficacy of using Oxygent treatment to reduce sickle cell RBC-induced vaso-occlusion in the *ex vivo* mesocecum vasculature of the rat.

PHASE II CLINICAL STUDIES

General surgery

Several Phase II studies using Oxygent in general surgery have been completed and have enrolled approximately 250 subjects (primarily orthopedic, urologic and gynecologic patients). Oxygent was well tolerated in these studies. In the first Phase IIa pilot study, the use of Oxygent was shown to increase blood oxygenation parameters (including mixed venous blood PO_2) in the absence of any adverse hemodynamic changes (Wahr *et al.*, 1996). In two large multicenter studies, Oxygent was dosed in conjunction with augmented-acute normovolemic hemodilution (A-ANH). This technique typically involves the collection of several (2–4) units of the patient's autologous blood just prior to surgery, administration of Oxygent to

replace the oxygen-carrying capacity of the harvested blood and blood lost during surgery, and later reinfusion of the collected autologous units when the surgery has been completed (Keipert and Stehling, 2002). By performing A-ANH, the patient's blood is temporarily diluted so that fewer red blood cells are actually lost during surgical bleeding.

As reported by Spahn *et al*. (1999), the European Phase II general surgery study in orthopedic surgery patients (n = 147) clearly demonstrated the drug activity of Oxygent in terms of rapidly enhancing the patient's systemic oxygenation status and being able to effectively reverse physiological transfusion triggers – i.e., protocol-defined physiological parameters indicating the patient's need for a blood transfusion. This study demonstrated a significant prolongation in the duration of trigger reversal in the Oxygent-treated patients, thereby effectively delaying the need for transfusion of allogeneic blood. Similar findings were found in a parallel US study performed in urologic and gynecologic surgery patients (n = 99), as reported in abstract form by Monk *et al*. (1998a, 1998b).

Cardiac surgery

Three small Phase II studies in cardiac surgery with Oxygent have enrolled ~80 patients undergoing coronary artery bypass grafting (CABG) procedures with CPB. In these studies a blood harvesting technique known as intraoperative autologous donation (IAD) was performed by the perfusionist just as bypass was initiated. By implementing aggressive pre-bypass harvesting of autologous blood in the Oxygent-treated patients, Hill *et al*. (2002) were able to demonstrate that Oxygent used in conjunction with IAD had the ability to prevent physiological transfusion triggers during bypass. More importantly, about 83 per cent of patients receiving the higher dose (2.7 g PFC/kg) of Oxygent were able to avoid transfusion of allogeneic blood completely (versus less than 45 per cent of control patients). By being able to tolerate a greater degree of autologous blood harvesting in the high-dose Oxygent group (~1600 ml vs ~990 ml in controls), these patients exhibited a trend (not significant due to the small number of patients per group) reduction in the allogeneic blood transfusion requirements from an average of 1.8 units/patient (range 0–7 units) in controls to only 0.4 units/patient (range 0–3 units).

PHASE III CLINICAL STUDIES

General surgery

A multicenter Phase III study in 492 patients undergoing orthopedic, urologic, abdominal, vascular and other major surgical procedures (often to treat malignant disease) was conducted at 34 medical centers in 8 European countries. As reported by Spahn *et al*. (2002), treated patients receiving Oxygent in conjunction with the A-ANH procedure avoided the need for blood transfusion more frequently than controls, and also required fewer units of allogeneic blood. The primary endpoint (reduction in RBC units transfused at 24 hours) was achieved in the intent-to-treat population (all randomized patients): the Oxygent group received 26 per cent fewer allogeneic units, 1.5 vs 2.1 units in controls (median 0 vs 1 unit; $P = 0.013$). By hospital discharge, the Oxygent group had received ~15 per cent fewer allogeneic units, (mean 2.7 vs 3.2 units; median of 1 vs 2 units), but this difference was no longer significant ($P = 0.16$). However, in the protocol-defined target population, i.e., patients with an estimated blood loss (EBL) $\geqslant$ 20 ml/kg (n = 330 or 67 per cent of randomized subjects), the Oxygent group required less RBC units (mean 2.0 versus 3.3 units; median 1 vs 3 units; $P < 0.001$) on postoperative day (POD) 1 (Table 28.1), and this difference remained significantly different from controls through day of discharge (DD) (mean 3.4 versus 4.9; median 2 vs 4 units; $P < 0.001$).

Regarding complete avoidance in the entire study population, ~53 per cent of patients in the Oxygent group avoided allogeneic blood transfusions compared to ~42 per cent of controls ($P < 0.05$) during the acute study period (24 hours). At later time points, more Oxygent-treated patients continued to avoid blood transfusions, but the difference versus controls was no longer significant. Again, however, in the protocol-defined target population (EBL $\geqslant$ 20 ml/kg), a significantly ($P < 0.05$) greater (almost two-fold) percentage of patients avoided transfusion at all time points from postoperative day 1 (D1) through postoperative day 21 or hospital discharge (P21/DD), whichever occurred sooner (see Figure 28.1).

For patients who had surgical blood losses ranging from ~10 ml/kg to >80 ml/kg (representing 86 per cent of all randomized subjects), the Oxygent benefit was highly statistically significant for both avoidance of blood ($P = 0.002$)

Table 28.1 Number of units of blood transfused from postoperative day 1 through hospital discharge

Study day	Oxygent group		Controls		% Reduction (Means)	*P*-value
Target pts (n = 330)	Mean* ± SD	Median	Mean* ± SD	Median		
1	2.0 ± 4.0	1	3.3 ± 3.0	3	40.8	< 0.001
3	2.7 ± 3.3	2	4.1 ± 2.7	3	33.2	< 0.001
7	3.2 ± 3.0	2	4.6 ± 2.5	4	30.3	< 0.001
21 or DD**	3.4 ± 2.9	2	4.9 ± 2.4	4	30.3	< 0.001

* Mean adjusted for covariates (analysis of covariance) using a natural log transformation.
** Whichever occurred sooner; DD is day of discharge.

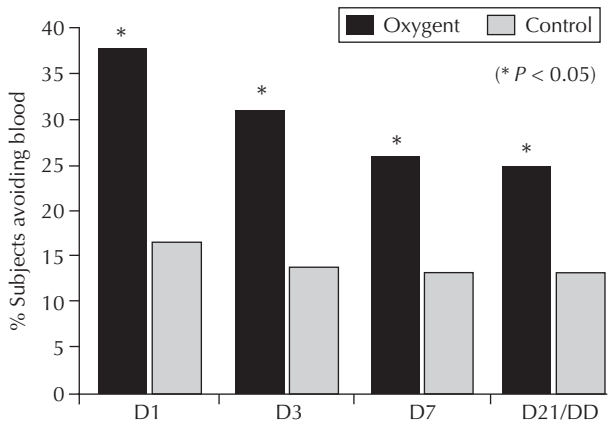

Figure 28.1 Avoidance of allogeneic blood transfusion from postoperative day 1 (D1) through postoperative day 21 or hospital discharge (D21/DD), in the protocol-defined target population (patients with EBL ⩾ 20 ml/kg).

and reduction in blood usage ($P < 0.001$), and this clinical benefit was maintained and remained statistically significant through 21 days or to the day of hospital discharge.

The safety profile in this study, evaluated by an independent Data Safety Monitoring Board (DSMB), was considered to be acceptable for further development of Oxygent, and the adverse events (AEs) observed were those expected following major elective surgery. The incidence of AEs was similar in the Oxygent group (86 per cent) compared to the control group (81 per cent). There was a higher overall incidence (~10 per cent differential) of serious adverse events (SAEs) in Oxygent-treated subjects (who had the A-ANH procedure) compared to controls (who did not). However, only the category 'Digestive system' was significantly different from control, mostly due to a higher reported occurrence of serious postoperative ileus (four instances in the Oxygent group versus none in controls). This reported 2 per cent incidence of ileus is rather low for large abdominopelvic operations, but surprisingly, the investigators did not report ileus in the controls – evidence that there was likely a reporting bias (i.e., in particular, underreporting of commonly encountered adverse events in the control group, since this was an unblinded study).

The DSMB monitoring this study did note group imbalances in certain adverse events, but concluded that there was no clinically consistent pattern or significance. They also concluded that since investigators were not blinded to treatment allocation, there was a possibility that this may have influenced reporting. The DSMB noted evidence that some investigators might not have adequately maintained normovolemia in the Oxygent-treated patients that were profoundly hemodiluted. Safety results from this study demonstrated that careful management of the patient's volume status and attention to optimal fluid balance is important to perform ANH safely. Overall mortality in this study was 3 per cent and the difference between groups (Oxygent 4 per cent vs controls 2 per cent) was not statistically significant. Tumor progression, sepsis and multiorgan failure, as well as typical surgical complications, were responsible for the deaths, and all were considered by the investigators to be due to underlying disease or conditions and were deemed to be unrelated to the study drug (Spahn *et al.*, 2002).

Cardiac surgery

In parallel to the general surgery study described above, a Phase III cardiac surgery study was conducted in primarily US and Canadian centers to assess transfusion avoidance in CABG patients undergoing CPB. In January 2001, after

approximately 410 patients (of 600 intended) had been randomized, enrollment in this study was voluntarily suspended when a statistically significant imbalance developed in the incidence of stroke. During the initial analysis of the safety data from these patients, an imbalance in thoracic bleeding events requiring re-operation was also uncovered.

Adverse events were reported for all subjects enrolled in this study, as expected in a CABG patient population. Protocol-specified analyses showed no statistically significant differences in the overall incidence of AEs or SAEs between groups. The incidence of SAEs (34.4 per cent overall; Oxygent 38.5 per cent; control 30.3 per cent) and deaths (1.5 per cent overall; Oxygent 2.5 per cent; control 0.5 per cent) were generally within published ranges expected up to 3 months post-CABG surgery involving CPB, although the 0.5 per cent mortality in the control group was exceptionally low. A cardiac surgery study published at that time by Klein *et al*. (2001), involving a large cohort of patients undergoing CABG surgery with CPB, reported a mortality of 4.5 per cent, which is similar to the 3.8 per cent mortality recently published by Pan *et al*. (2004) in a study of over 1660 patients undergoing primary CABG surgery at the Texas Heart Institute between January 2000 and December 2001.

Although the overall intraoperative and postoperative safety findings in this study appeared to be acceptable, there was a significantly higher incidence of serious neurological events, including primarily strokes (2.4 per cent overall; Oxygent 5 per cent vs control 1 per cent). As it was for mortality, the incidence of neurological events in the controls was lower than expected for a CABG patient population. An imbalance in the incidence of serious postoperative thoracic chest tube bleeding requiring re-operation, a noted postsurgical complication after CABG surgery, also was observed (5.8 per cent overall; Oxygent 10 per cent vs control 1.5 per cent). Once again, the incidence of this SAE in the control group in this unblinded study was remarkably low compared to that reported in the literature. The overall incidence rates for the serious neurological and bleeding complications observed were within clinical expectations, and within ranges reported in the literature.

The etiology of the higher rates of these complications in subjects randomized to the Oxygent group compared to controls was the subject of extensive *post hoc* exploratory analyses and hypothesis generation. After detailed analyses of safety data from this study and all previous clinical studies, as well as additional laboratory evaluation of Oxygent in the presence of compounds that would come into contact with the emulsion in the bypass setting, no evidence was found to link Oxygent directly to the observed imbalances. Primary contributing factors for the adverse events appear to be the amount of autologous blood harvested and the way in which the rapid IAD procedure was performed in the Oxygent-treated group. In addition, there were imbalances in some of the risk factors for these events between the treatment and control groups, and a greater degree of dilutional coagulopathy and use of hetastarch in the Oxygent group. The result was that, in a subset of subjects at greater risk for complications, inadequate management of blood pressure during rapid blood harvesting potentially resulted in decreased perfusion to the brain. The conclusion that the procedure, rather than the product, was responsible for the adverse events was supported by the fact that there have been no similar imbalances in the incidence of stroke in the three Phase II cardiac surgery studies (in which neurological and neuropsychiatric evaluations were performed in a blinded manner). No similar imbalance was seen in the Phase III general surgery studies, or in any previous clinical studies with Oxygent. All of these findings were summarized in a Clinical Information amendment and an Integrated Summary of Safety (ISS) and were subsequently provided to the FDA and to the European regulatory authorities (EMEA).

As reported recently by Frumento *et al*. (2002), a small subset of patients in the Phase III cardiac surgery study were being monitored with gastric tonometry to assess the overall status of gut perfusion and oxygenation. As shown in Figure 28.2(A), a significant benefit was seen in the Oxygent-treated patients; the CO_2 gap (i.e., gastric CO_2 minus $PaCO_2$) was significantly lower ($P < 0.001$) and gastric pH was significantly higher ($P < 0.01$), suggesting improved perfusion of the gut. These benefits, both of which persisted throughout surgery, suggested better maintenance of gut microcirculatory oxygenation and translated into significantly ($P < 0.007$) faster return of normal bowel function in the postoperative period (Figure 28.2(B)).

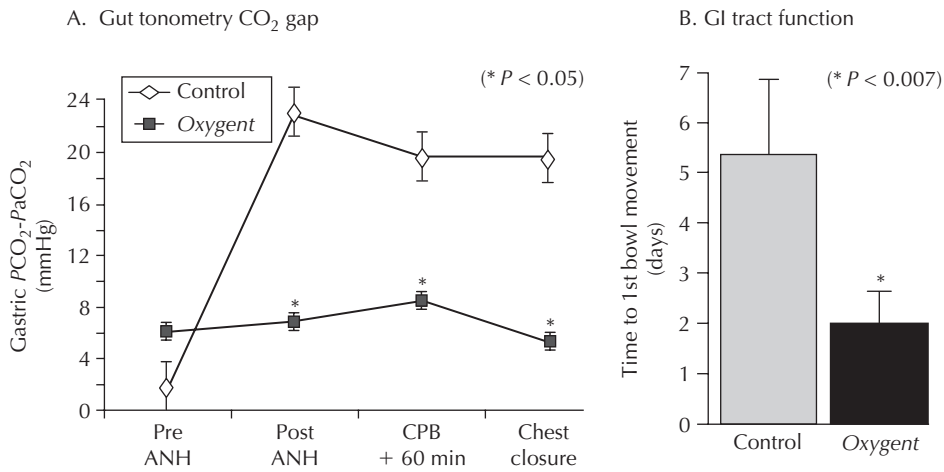

Figure 28.2 Assessment of gut perfusion/oxygenation status and bowel function. (A) CO_2 gap (gastric CO_2 measured by tonometry minus $PaCO_2$). (B) Postoperative recovery of gut function determined by time to first bowel movement.

FUTURE CLINICAL DEVELOPMENT

Avoidance of allogeneic blood transfusion remains a highly desired clinical objective of both physicians and patients. Previous clinical studies in surgery have demonstrated that using Oxygent to augment the amount of autologous blood harvested could result in a significant decrease in allogeneic transfusion requirements, primarily in patients who lose at least 3 units of blood during surgery. However, careful analysis of safety data from these studies also indicated that ANH, and especially the rapid IAD just before cardiac bypass, must be performed very carefully, with close attention to optimal blood volume management. Hence, based on the data from their previous Phase II and Phase III clinical trials, Alliance designed a new Phase III protocol without any autologous blood harvesting to further evaluate the efficacy and safety of Oxygent in a general surgery population. Oxygent would be administered when surgical bleeding had decreased the patient's hemoglobin to a level that results in a physiologic or Hb-based transfusion trigger. Delaying Oxygent dosing until a trigger was reached would ensure that patients only received Oxygent therapeutically (i.e., when transfusion is clinically warranted), thereby allowing the drug to be evaluated in a manner consistent with the way blood transfusions are typically administered.

Alliance submitted this new Phase III protocol to the European Agency for the Evaluation of Medical Products (EMEA) in 2004, to seek formal scientific advice regarding development of Oxygent as an alternative to blood transfusions in elective surgery. Contrary to previous informal guidance received from regulatory authorities in key European countries (including France, the Netherlands and the UK), the EMEA indicated that if avoidance of blood transfusion were to be pursued as a primary endpoint the safety comparison would be complex, and suggested that Alliance consider an initial indication that would not require a direct comparison to allogeneic blood transfusion. Alliance requested further clarification of this opinion, which made it clear that the EMEA did not believe it was feasible at the present time to conduct a study that was of sufficient size to demonstrate equivalent safety of an oxygen therapeutic with donor blood, based on the low incidence of serious adverse effects known to be associated with transfusion of allogeneic blood (e.g. death, and transmission of viral diseases caused by HIV and hepatitis contamination). Due to this regulatory opinion from the EMEA, Alliance has decided that it will be necessary to pursue an alternative clinical indication for the initial commercialization of Oxygent, namely, specifically to exploit the efficient oxygen delivery capability of Oxygent to enhance tissue oxygenation and protect organs from ischemic injury, thereby potentially decreasing postoperative complications arising from acute tissue hypoxia during elective surgery.

Since late 2002, following the setback in clinical development because of the safety findings in their Phase III CABG trial, Alliance have had to downsize the company and terminate all development work on Oxygent due to lack of funding and the dissolution of a joint venture licensing agreement with Baxter Healthcare. More recently, throughout 2004, Alliance has been actively engaged in licensing discussions with potential pharmaceutical partners willing to provide the necessary resources to complete the remaining clinical and regulatory development necessary for future commercialization of Oxygent in Europe and in Asia. In addition, efforts continue to secure sufficient new financing that would support contract manufacturing, and the remaining clinical development needed for future commercialization of Oxygent in North America.

In addition to elective surgery, a number of other possible applications exist for Oxygent that may be pursued in the future. These include trauma resuscitation and urgent situations where donor blood is necessary for transfusion but not immediately accessible. Oxygent could provide tissues with immediate oxygenation to delay transfusion until a definitive need for blood can be established, or could provide a temporary 'oxygenation bridge' to stabilize patients until blood becomes available. Future medical indications may focus on augmentation of tumor oxygen levels to enhance sensitivity to radiation and chemotherapy, as a treatment for reversing sickle cell crisis, as a post-dive treatment for decompression sickness, and for preservation of tissues and organs destined for transplantation.

EDITOR'S SUMMARY

Oxygent, like Oxyfluor, is a 'second-generation' perfluorocarbon-based emulsion. Oxygent contains 60 g of the perfluorochemical perflubron per 100 ml of emulsion, and also contains a small quantity of perflubrodec (perfluorodecyl bromide; $C_{10}F_{21}Br$), added as a stabilizer. It is emulsified with egg-yolk phospholipid (EYP). The concentration and oxygen capacity of Oxygent is approximately three times that of Fluosol and Perftoran. The formulation and manufacturing of Oxygent was optimized, and the stability of the emulsion enabled it to be stored for long periods of time. Early formulations of Oxygent induced transient inflammatory responses in animals and humans, found to be related to particle size. Subsequent formulations overcame these side effects. Phase I and II clinical studies showed both safety and efficacy, but safety concerns were raised in a pivotal Phase III cardiac surgery study in which patients who received Oxygent experienced strokes significantly more frequently than control patients. The developer of Oxygent, Alliance Pharmaceutical Corp., attributed these results to defects in design of the clinical trials and procedures for the use of Oxygent, rather than to inherent properties of the emulsion. Nevertheless, economic pressures forced cessation of clinical development.

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SECTION

6

Hemoglobin-Based Oxygen Carriers

In the case of hemoglobin-based oxygen carriers, oxygen is bound to the carrier (hemoglobin) – in contrast to the perfluorocarbons, which carry oxygen in solution. The difference is that when bound, release to tissue is governed, at least in part, by the release from hemoglobin, so oxygen will be delivered differentially to tissue according to the characteristics of the hemoglobin. This is similar to the way in which red blood cells deliver oxygen, which explains why, historically, hemoglobin-based products have been appealing. This section describes the hemoglobin molecule and ways in which it can be modified, and then presents experience with each of the classes of molecules (crosslinked, polymerized and conjugated) that have reached clinical trials so far. Recombinant hemoglobin is discussed, along with the potential this technology promises for exploration of the remaining problems in developing a safe and efficacious blood substitute.

Chapter 29

The Structural and Functional Properties of Hemoglobin and their Relevance for a Hemoglobin-Based Blood Substitute

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INTRODUCTION

Hemoglobin (Hb) and myoglobin (Mb) were the first proteins whose three-dimensional structure was discovered, by M. F. Perutz and J. C. Kendrew, at atomic resolution by X-ray crystallography. Both proteins contain iron-protoporphyrin IX (the heme) as the prosthetic group that reversibly coordinates O_2 and several other gaseous and non-gaseous ligands. Mb is constituted by a single polypeptide chain, whereas Hb is a tetramer constituted by two different subunits named α and β, with the quaternary formula $\alpha_2\beta_2$. Mb and the α and β chains of Hb share a common fold, the so-called *globin-fold*, and are strongly correlated from an evolutionary standpoint (Antonini and Brunori, 1971; Dickerson and Geis, 1983).

All vertebrates and many invertebrates have genes for Hb and Mb, or at least for globin-like hemoproteins. The human genome contains the genes for several hemoglobin chains; however, out of the 320 mg/ml Hb (5 mM per tetramer or 20 mM per heme) contained in the red blood cells of adult, healthy humans, approximately 98 per cent is accounted for by HbA ($\alpha_2\beta_2$) (Surgenor, 1974), and thus we feel justified in limiting our attention to this protein. During fetal life human embryos and fetuses produce other Hbs, specialized for O_2 uptake from the placenta (Dickerson and Geis, 1983; Forget *et al.*, 2001), but these will not be described in this review. Many genetic variants of fetal and adult Hb are known, some of them pathological and other asymptomatic: the work *A Syllabus of Human Haemoglobin Variants* (Huisman *et al.*, 1996) lists 199 variants for the α chains and 335 for the β chains. Moreover, genetic mutations in the non-coding region of the hemoglobin genes may result in the unbalanced synthesis of otherwise normal chains, intracellular precipitation of the excess chain and destruction of newly formed red cells in the bone marrow; the resulting anemias are called thalassemias (Surgenor, 1974; Forget *et al.*, 2001).

In view of the detailed knowledge on structure–function relationships in Hb, it is often possible to trace specific functional properties to single amino-acid residues in the polypeptide chains; therefore genetic engineering has been applied to human Hb in order to synthesize protein variants more suitable than the wild type for transfusional applications. Some of these 'hot' positions of the amino-acid sequence will be reviewed below. The huge amount of literature on Hb makes it extremely difficult to summarize in a few pages even the very general aspects of

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the structure, function, dynamics and evolution of Hb; the interested reader is referred to books and reviews (e.g. Antonini and Brunori, 1971; Imai, 1982; Emerse *et al.*, 1994; Bunn and Forget 1986; Dickerson and Geis, 1983).

THE STRUCTURE OF HEMOGLOBIN

The 'globin fold' characteristic of Mb and Hb is built by α-helices which provide a deep crevice where heme is bound, as shown schematically in Figure 29.1. Mb and the β subunits of Hb contain eight helices, while the α subunits have only seven. The helices are indicated by the letters A through H starting from the amino terminus; junctions between helices may either be direct or via short non-helical segments – the CD, EF, FG and GH corners. The α chain lacks the short helix D, and the junction between helices C and E is provided by the CE loop. Each amino acid residue is indicated by its position in the sequence and its topology with respect to the α-helix to which it belongs. This notation has the advantage of an easier comparison among different globins – for example, the invariant His that coordinates the heme iron occupies the eight position of the F helix (F8) and corresponds to residue number 93 in sperm whale Mb, 87 in the α chain and 92 in the β chain of HbA (Perutz, 1970; Dickerson and Geis, 1983).

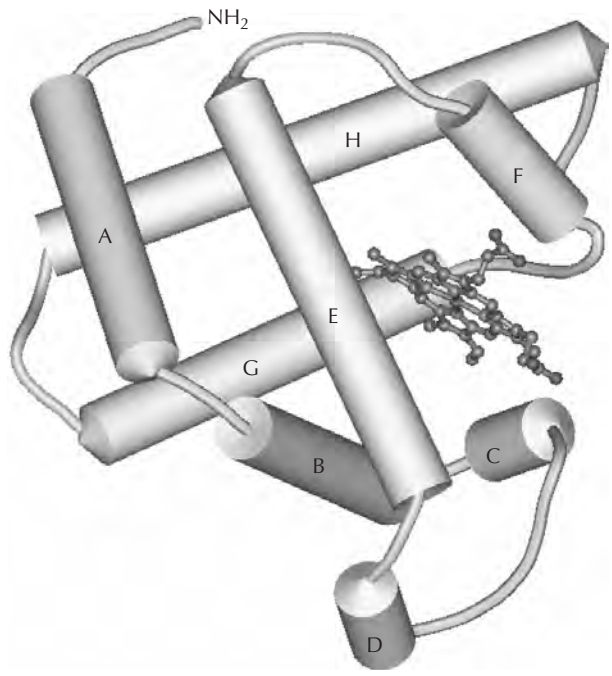


Figure 29.1 The 'globin fold' as exemplified by sperm whale myoglobin. The eight α-helices are named A through H. See color plate 5.

The hydrophobic heme-binding pocket is provided mainly by helices E, F and G, and the FG and CD (or CE) corners. Opposite to the G helix, the heme's propionates face the solvent (Figure 29.1). The tight shelter provided to the heme limits its communication with the bulk, a fact that may appear paradoxical; however, limiting exposure of the ferrous heme to solution components is essential to reduce the rate of oxidation to the physiologically incompetent ferric (or met) state (see below). As a result of the burial of the heme in the protein matrix, there appears to be no room for O_2 to diffuse to the heme iron; however, the flexibility of the protein clears a path through the concerted movements of Histidine E7 and Arginine CD3. To achieve this result, the whole protein dynamics is necessary (Elber and Karplus, 1990).

The heme ring does not establish covalent bonds with the protein and is wedged in place by weak interactions, among which that with Phenylalanine CD1 (or CE1) is the most conserved; the heme iron is coordinated by the highly conserved Histidine F8 (the proximal histidine). Due to its planar shape and large diameter, the heme effectively divides the cavity into a 'proximal' pocket on the side of Histidine F8, and a 'distal' pocket where O_2 and other ligands are accommodated. In the distal pocket, a second largely conserved histidine residue (the distal Histidine E7) establishes a hydrogen bond with O_2 and plays important physiological roles in discriminating between ligands (e.g. O_2 and CO), providing stability to the O_2 adduct and gating the access to the iron (Perutz, 1979; Olson *et al.*, 1988).

DERIVATIVES WITH HEME LIGANDS

Ferrous (Fe^{2+}) Hb with no ligand on the distal side of the heme is called unligated (or deoxy) Hb. It may be prepared by removal of the heme ligand from the corresponding ferrous ligated form (such as O_2, CO, NO, alkylisocyanides, and nitroso aromatic compounds, NO being the strongest and bulky alkylisocyanides the weakest ligands). Usually the starting derivative is oxygenated Hb (oxyHb or HbO_2), and deoxygenation is achieved upon exposure to vacuum, equilibration with an inert gas (e.g. N_2, Ar) or addition of sodium dithionite ($Na_2S_2O_4$), which removes O_2 from solution. DeoxyHb may also

be prepared by reduction of the ferric derivative using borohydride, dithionite, ascorbate, or other reductants (Antonini and Brunori, 1971).

The Fe^{2+} is subject to slow spontaneous oxidation in the presence of O_2, yielding ferric (Fe^{3+}) or metHb. In blood the rate of autoxidation is about 3 per cent per day, but the oxidized iron-porphyrin is reduced to its active form by a reductase (see below). The ferrous derivatives are oxidized rapidly by ferricyanide or by nitrite. Upon oxidation, the heme iron coordinates a water molecule that can be replaced by F^-, OCN^-, SCN^-, N_3^-, imidazole, or CN^-, where F^- is the weakest and CN^- the strongest ligand (Antonini and Brunori, 1971).

An endogenous ligand, such as the imidazole of His E7, may also bind to the ferric iron under exceptional conditions. In this hexacoordinate bis-histidine complex, the fifth and sixth axial positions are occupied by the proximal and distal histidines, respectively, yielding a low spin derivative called a hemichrome. Denaturation of the protein may cause the formation of other hemichromes, e.g. that with the sulfur of Cysteine (93)F9β. Addition of dithionite and reduction of heme iron leads to formation of low spin compounds called hemochromes.

SUBUNIT INTERFACES AND TETRAMER–DIMER DISSOCIATION

The quaternary structure of Hb is of utmost importance to its function (Perutz, 1970; Baldwin and Chothia, 1979; Dickerson and Geis, 1983). The tetramer has a symmetry axis and can be considered as a dimer of αβ dimers, called the $\alpha_1\beta_1$ and $\alpha_2\beta_2$. The $\alpha_1\beta_1$ dimer (and the symmetrical $\alpha_2\beta_2$) may be considered the basic structural unit, and dissociation of the tetramer into dimers involves cleavage along the symmetric interfaces $\alpha_1\beta_2$ and $\alpha_2\beta_1$ (Antonini and Brunori, 1971; Ackers and Johnson, 1981). The overall dissociation into subunits may be represented as follows:

$$\alpha_2\beta_2 \leftrightarrow 2\alpha\beta \leftrightarrow 2\alpha + 2\beta$$

In solution, the α subunits are mostly monomers while the β subunits aggregate into the β_4 homotetramer (Antonini and Brunori, 1971).

Deoxy and liganded Hb have a different quaternary structure (Perutz, 1970; Baldwin and Chothia, 1979) that can be represented as a translation and a rotation of the relative position of the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers around a pivot (as shown in Figure 29.2). The dissociation constant of the tetramer into the dimers is very strongly affected by the ligation state of Hb; thus HbO_2 dissociates into αβ dimers with $K_d = 2.4\,\mu M$/heme, whereas deoxyHb has $K_d = 44\,pM$/heme (at pH = 7.4 and T = 21.5°C; see Ackers and Johnson, 1981). The dissociation into dimers causes extracellular Hb to be filtered by the kidney, as it occurs in hemolytic crisis; in the case of transfused Hb, this shortens the lifetime of the protein in the circulatory tree and causes renal toxicity.

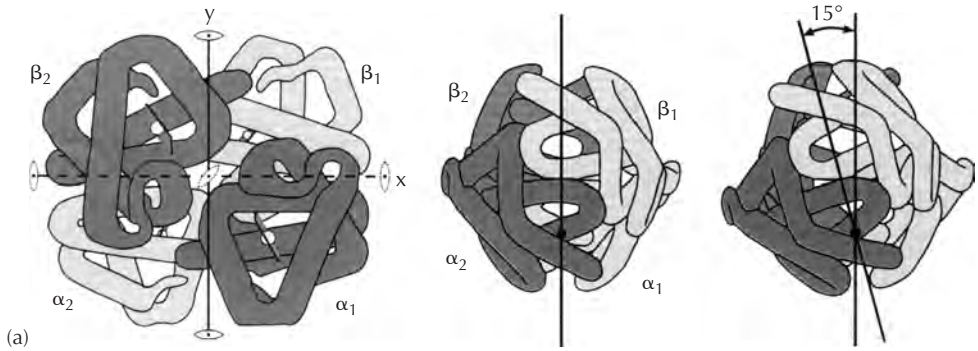
The interdimeric $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces involve mainly the α subunits of one dimer and the β subunits of the other (see also Figure 29.2); they are contributed by residues from helix C and the FG corner of both subunits. Principal contact areas involve side chains from the αFG-βC and the αC-βFG regions as well as less relevant contacts at αFG-βFG and αC-βC. Minor contacts between the two α chains, and a loosely packed interface between the two β chains (seen only in oxyHb) may be mentioned. The total surface contact area of the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces corresponds to $3090\,A^2$ per tetramer in the deoxy quaternary state and $2950\,A^2$ in the oxy quaternary state (Lesk *et al.*, 1985).

The $\alpha_1\beta_1$ interface is contributed by the outer surface of helices B, G and H of both subunits plus helix D from the β subunit. It involves 19 amino acid residues from both the α and the β chains, and corresponds to a buried surface area of $\sim 1700\,A^2$ per dimer (Chothia *et al.*, 1976; Lesk *et al.*, 1985). The three main contact regions are: αB-βH; αG-βG and αH-βB. The $\alpha_1\beta_1$ interface is very stable, and the dissociation constant of dimers into subunits has been estimated at $K_d = 9.4 \times 10^{-13}$ M per heme (Shaeffer *et al.*, 1984; Mrabet *et al.*, 1986). In the presence of a reagent of the sulfhydril groups such as p-chloro mercurybenzoate (PMB), all the Cysteine residues (G11α, F9β and G14β) are reacted and reassociation is inhibited. This reaction allowed Bucci and Fronticelli (1965) to purify the α and β chains of human Hb; upon removal of PMB, the subunits reassembled spontaneously into functional tetramers.

COOPERATIVE OXYGEN BINDING

The oxygen equilibrium curve

Oxygen binding by Mb and the isolated Hb chains, irrespective of their aggregation state, conforms



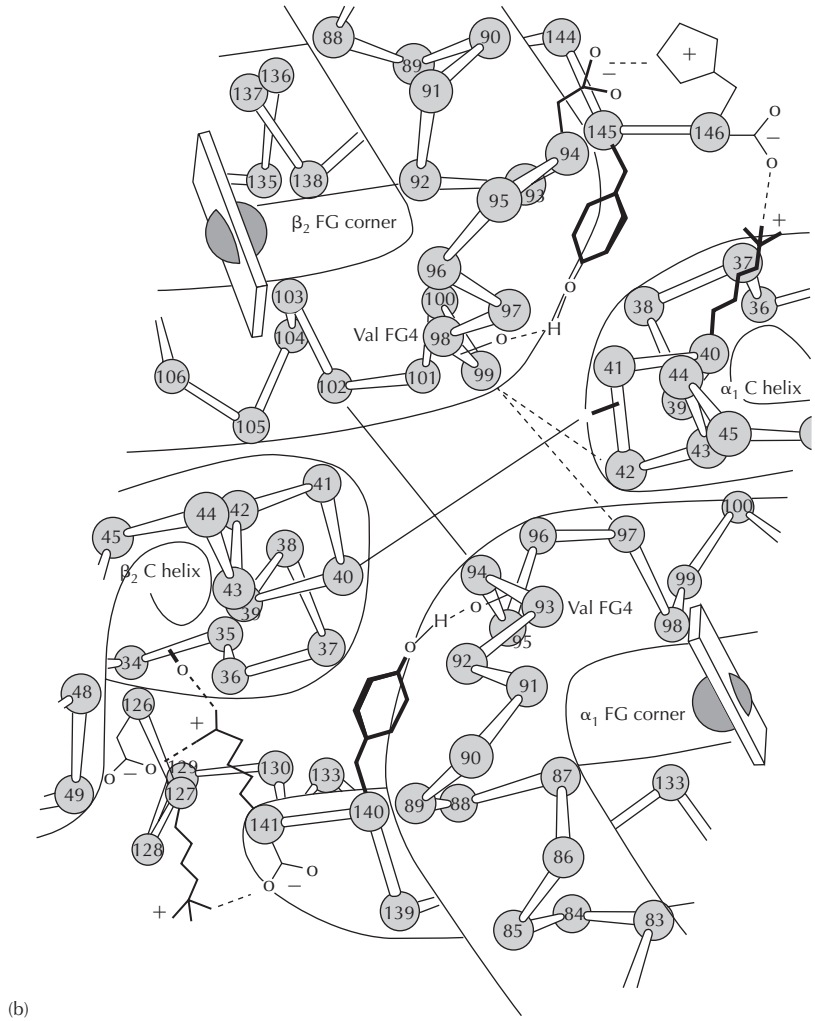


Figure 29.2 The allosteric transition in HbA. (a) Overview of the allosteric transition (reproduced with permission from Eaton *et al.*, 1999). The allosteric transition consists mainly of a sliding and a rotating motion of the $\alpha_1\beta_1$ dimer with respect to the $\alpha_2\beta_2$, with a consequent rearrangement of the $\alpha_1\beta_2$ interface and the symmetric $\alpha_2\beta_1$. (b) Details of the amino acid contacts at the $\alpha_1\beta_2$ interface (modified from Dickerson and Geis, 1983). See color plate 4.

Table 29.1 Affinity of the gaseous ligands of sperm whale Mb and human HbA

Protein	Ligand	Conditions			k′	*n*	k′	k	Ref.
		pH	T	Effectors	(μM^{-1})		($\mu M^{-1}s^{-1}$)	(s^{-1})	
Mb	O_2	7.0	20°C	–	1.50	1.0	15	10	1
Mb	CO	7.0	20°C	–	33.3	1.0	0.50	0.015	1
Mb	NO	7.0	20°C	–	(1.4×10^5)	(1.0)	17	1.2×10^{-4}	2
HbA (overall)	O_2	9.1	25°C	0.1 M Cl^-	0.26	2.73			3
HbA (overall)	O_2	7.4	25°C	0.1 M Cl^-	0.11	3.02			3
HbA (overall)	O_2	6.5	25°C	0.1 M Cl^-	0.03	2.88			3
HbA (overall)	O_2	7.4	25°C	5 mM Cl^-	1.07	2.51			4
^{T}HbA	O_2	7.4	25°C	5 mM Cl^-	0.065	1.0			4
^{R}HbA	O_2	7.4	25°C	5 mM Cl^-	2.42	1.0			4
HbA (overall)	CO	6.94	25°C	10 mM IHP	3.89	2.8			5
HbA (overall)	NO	7.0	20°C	0.05 M phosphate	–	(1.6)	26	0.95×10^{-5} 8×10^{-4}	2
Isolated α chains	O_2	7.0	20°C	–	1.79	1.0	50	28	1
Isolated α chains	CO	7.0	20°C	–	308	1.0	4.0	0.013	1
Isolated α chains	NO	7.0	20°C	–	(5.2×10^5)	(1.0)	24	4.6×10^{-5}	2
Isolated β chains	O_2	7.0	20°C	–	3.75	1.0	60	16	1
Isolated β chains	CO	7.0	20°C	–	563	1.0	4.5	0.008	1
Isolated β chains	NO	7.0	20°C	–	(1.1×10^6)	(1.0)	24	2.2×10^{-5}	2
α chains in ^{T}HbA	O_2	7.0	20°C	–	0.016	1.0	2.9	183	1
α chains in ^{T}HbA	CO	7.0	20°C	–	1.0	1.0	0.1	0.1	1
β chains in ^{T}HbA	O_2	7.0	20°C	–	0.0047	1.0	11.8	2500	1
β chains in ^{T}HbA	CO	7.0	20°C	–	1.0	1.0	0.1	0.1	1
α chains in ^{R}HbA	O_2	7.0	20°C	–	4.92	1.0	59	12	1
α chains in ^{R}HbA	CO	7.0	20°C	–	650	1.0	6.5	0.01	1
β chains in ^{R}HbA	O_2	7.0	20°C	–	2.81	1.0	59	21	1
β chains in ^{R}HbA	CO	7.0	20°C	–	650	1.0	6.5	0.01	1

k = dissociation rate constant; k′ = association rate constant; K = the equilibrium constant (K = k′/k). Values in parentheses are calculated rather than experimentally determined. References: 1, Mims *et al.*, 1983; 2, Moore and Gibson, 1976; 3, Imai, 1982 (values of K were calculated from the *P*50 using a solubility coefficient of 1.78 μM/mmHg); 4, Imai, 1973; 5, Di Cera *et al.*, 1987.

to a simple chemical equilibrium, i.e., the fraction of oxygenated hemes (Y) is a hyperbolic function of O_2 partial pressure (PO_2) (Antonini and Brunori, 1971). Binding of all other ligands to Mb and the isolated chains is likewise a simple hyperbolic equilibrium; however, the affinity constants vary widely (see Table 29.1).

The O_2 affinity of hemoproteins is regulated by the interactions established by the protein with the heme and the bound gas; we define this type of regulation the tertiary or intrinsic control. In Hb, the quaternary assembly constrains the structure of the subunits and adds a second level of O_2 affinity regulation, the quaternary or allosteric control. The tertiary control of O_2 affinity is exerted mainly by residues on the proximal and distal sides. The proximal Histidine F8 contributes to pull the iron out of the heme plane, causing its doming; the stronger this effect, the lower the ligand affinity (Perutz, 1979; Perutz *et al.*, 1998). The distal Histidine E7 stabilizes the bound O_2 via a hydrogen bond, and mutations of this residue invariably lower the O_2 affinity by increasing its dissociation rate constant (see below) (Olson *et al.*, 1988).

In Hb, the O_2 dissociation curve is sigmoidal rather than hyperbolic, which is evidence for heme–heme cooperative interactions (Antonini and Brunori, 1971; Wyman and Gill, 1990). As a consequence of cooperativity, the affinity of Hb for O_2 increases as Y increases, the first O_2 molecule being bound with the lowest affinity. This phenomenon makes it difficult to describe the O_2 binding isotherms in a model independent way. A useful way of analyzing the O_2 dissociation curve is based on the empirical Hill equation:

$$Hb + nO_2 \leftrightarrows Hb(O_2)_n;\ K = \frac{[Hb(O_2)_n]}{[Hb]p^n} \qquad (29.1)$$

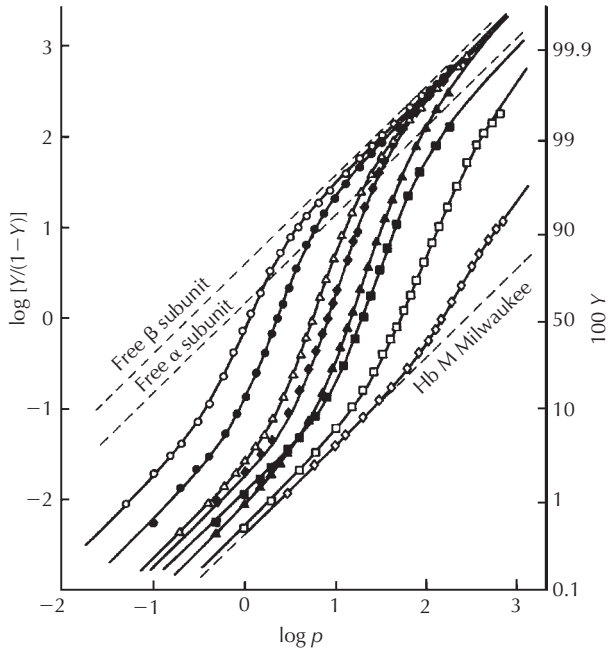


Figure 29.3 Oxygen binding isotherms for HbA under some representative experimental conditions; temperature 25°C, hemoglobin concentration 0.6 mM per heme, for all data sets (reproduced with permission from Imai, 1982). The binding isotherms of the isolated α and β chains (that mimic the affinity of ^{R}Hb) and of the natural mutant Hb Milwaukee (that presents a strongly stabilized T state) are reported for comparison. Symbols: circles, 0.05 M tris buffer pH = 9.1, chloride concentration 2.6 mM (open circles) or 0.1 M (closed circles); triangles, 0.05 M bistris buffer pH = 7.4 containing 0.1 M chloride, 2,3-DPG concentration 0 (open triangles) or 2 mM (closed triangles); closed diamonds, 0.05 M bistris buffer pH = 7.4 containing 0.1 M chloride and 5% CO_2 in the gaseous phase throughout; open squares, 0.05 M bistris buffer pH = 7.4 containing 0.1 M chloride and 2 mM IHP; closed squares, 0.05 M bistris buffer pH = 6.5 containing 0.1 M chloride; open diamonds, 0.05 M bistris buffer pH = 6.5 containing 0.1 M chloride and 2 mM IHP.

where O_2 activity is expressed in terms of its partial pressure *P*. In logarithmic terms, this becomes:

$$log \frac{Y}{1-Y} = log K + n log p \qquad (29.2)$$

Although the original physical meaning of the Hill equation has been disproved, it is still used since it describes the experimental data with only two parameters: (1) *P*50, i.e., the O_2 partial pressure required to yield half-saturation of hemes (P value at Y = 0.5), and (2) *n*, the slope in the Hill plot (see Figure 29.3), which is related to cooperativity (the higher its value above 1, the higher the cooperativity). A value of $n = 1$ (as found for the β_4 homotetramer) corresponds to a hyperbolic binding curve, indicating equivalence of hemes and absence of cooperative interactions. Observation of $n < 1$ is not proof of negative cooperativity (i.e., that binding of a ligand diminishes the affinity for subsequent binding), since it can also arise from various kinds of heterogeneity, intramolecular (e.g. differences in affinity between α and β subunits) or intermolecular (e.g. a mixture of various Hbs). Typically, the Hill coefficient *n* is the same for all ligands of ferrous Hb in spite of their *P*50 (or C_{50} for non-gaseous ligands) varying some 200 000-fold, and it ranges between 2.6 and 3.0 over a wide array of experimental conditions (Antonini and Brunori, 1971; Imai, 1982).

Structural basis of cooperativity

The structural basis of cooperativity lies in Hb being stable in two distinct quaternary structures. O_2 binding causes the protein to switch from the low affinity conformation (T state) characteristic of unliganded Hb to the high affinity one (R state), characteristic of oxyHb (see Figure 29.2). The thermodynamic aspects of cooperativity were described by Monod *et al*. (1965), whereas the structural basis of allostery was unravelled by Perutz (Perutz, 1970; Perutz *et al*., 1998); an attempt to correlate structure and thermodynamics is due to Szabo and Karplus (1972). Cooperativity is expressed either in the combination or the dissociation rate constants for the different ligands of ferrous Hb, the contribution of either one varying considerably (Szabo, 1978). An important early observation, inconsistent with Hill's equation but fully consistent with the allosteric model, is that the Hill plot presents an upper and a lower asymptote with $n = 1$ (see Figure 29.3). These asymptotic values represent the non-cooperative O_2 binding to ^{T}Hb and ^{R}Hb; hence their span is proportional to the cooperative free energy (Wyman and Gill, 1990).

Cooperativity has physiological relevance, since it causes the O_2 binding curve to be steeper around half saturation, thus maximizing O_2 release at relatively high O_2 partial pressures

(Forget *et al.*, 2001). We may summarize the basis of O_2 transport by Hb by comparison with (1) O_2 transport by plasma and (2) O_2 transport by a hypothetical non-cooperative O_2 carrier. The gas physically dissolved in the plasma contributes only 2 per cent to the total blood O_2, thus O_2 content is low even when PO_2 is relatively high. Oxygen carriers, be they cooperative as Hb or non-cooperative as Mb, invert this condition and yield high O_2 content even at relatively low PO_2 values; however, a non-cooperative O_2 carrier with affinity high enough to saturate in the lungs would require very low tissue PO_2 to release a significant fraction of the bound gas.

In spite of their importance, precise estimates of the O_2 affinities of ^{T}Hb and ^{R}Hb under physiological conditions are still debated, the reason being that these parameters are strongly correlated to each other and to the equilibrium constant of the allosteric transition (see below) (Imai, 1982; Wyman and Gill, 1990). A reasonable compromise is as follows: K_T depends on the experimental conditions, with special reference to the presence of allosteric effectors, and may vary between 5 and over 200 mmHg of O_2 (Yonetani *et al.*, 2002); K_R under non-extreme experimental conditions may be assimilated to that of the isolated chains ($\sim$0.2 mmHg). The equilibrium constant of the allosteric transition is defined as $L = [^T\text{Hb}]/[^R\text{Hb}]$. The value of L, which is influenced by the presence of heme and non-heme ligands, is quite difficult to determine due to its strong correlation with K_R. Imai (1973) reported the following values for the allosteric constant: for deoxyHb at physiological pH, $L_0 = 3900$, while in fully oxygenated HbO_2 $L_4 = 0.002$.

Models of cooperativity

Cooperativity was recognized early on as a challenge to thermodynamics, since it implies that in Hb high and low affinity sites co-exist and that the former combine after the latter, rather than *vice versa*. Hill proposed that the stoichiometry of the reaction between O_2 and Hb is described by the non-integer *n*, suggested to correspond to the average polymer size of Hb in solution; but this hypothesis was disproven by Adair (1925), who demonstrated that Hb is a stable tetramer. Adair correctly stated that all ligation intermediates are present in solution and that the equilibrium between Hb and O_2 is described by four equilibrium constants, whose increase is responsible for cooperativity; since then, the challenge has been to explain the progression of the Adair's constants. Leaving aside the numerous sequential models (see Koshland *et al.*, 1996), which only have a historical interest, a major breakthrough came with Monod's proposal that the explanation of cooperativity was to be found in allostery. The allosteric model (Monod *et al.*, 1965) postulated that two conformations are accessible to Hb, named *T* for tense and *R* for relaxed, and that these are in equilibrium even in the absence of ligands. The crystallographic structures that M. F. Perutz was discovering at the same years provided strong support to the allosteric theory, since they demonstrated two (and, we may add after over 40 years, only two) quaternary conformations of Hb.

The postulates of the allosteric model are as follows: (1) Hb is a symmetric tetramer in both allosteric conformations, i.e., all subunits are either *T* or *R*, and mixed or intermediate conformations are not allowed; and (2) the protein in the absence of ligands populates the low affinity *T* conformation, whereas when liganded it prefers the high affinity *R* conformation; this implies that the allosteric equilibrium constant L_0 ($= [^T\text{Hb}]/[^R\text{Hb}]$) is greater than unity, whereas L_4 ($= [^T\text{Hb}(O_2)_4]/[^R\text{Hb}(O_2)_4]$) is lower than unity. These postulates demand that dimers and the isolated chains have the ligand affinity of ^{R}Hb (i.e., the quaternary assembly constrains reactivity), a prediction that was brilliantly fulfilled. The schematic representation of cooperativity in a free energy diagram (Shulman *et al.*, 1975) is depicted in Figure 29.4.

STEREOCHEMICAL CONSEQUENCES OF LIGAND BINDING

Upon ligand binding, important stereochemical changes occur at the heme. The iron of unliganded heme is five-coordinate, by the pyrrole nitrogens and the proximal Histidine F8; its geometry is square-pyramidal, and results in the characteristic doming of the heme (Perutz, 1979). After ligation, the six-coordinate iron assumes a more or less symmetric octahedral geometry and the heme flattens, pulling the proximal Histidine F8 and the F helix and FG corner with it. These parts of the macromolecule constitute the so-called 'allosteric core' of Hb and Mb (Gelin *et al.*, 1983). In Mb these structural changes have no further consequences, but in Hb the FG corner is part of the $\alpha_1\beta_2$ interface and its movement triggers the allosteric transition

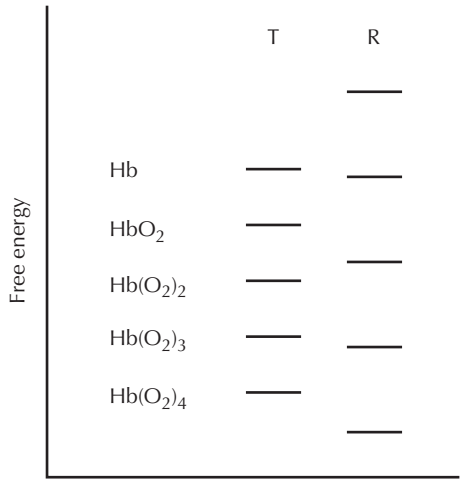


Figure 29.4 Free energy diagram of the two-state allosteric model of Monod *et al*. (1965). The ligand binding free energy of ^{T}Hb is smaller than that of ^{R}Hb, but ^{T}Hb is favored by (RT ln L_0); hence in the absence of oxygen ^{T}Hb is prevalent; however, each successive molecule of oxygen that binds favors ^{R}Hb, until this allosteric state becomes prevalent (usually between the second and the third molecules of oxygen).

(Figure 29.2). Extensive differences between ^{T}Hb and ^{R}Hb are observed at the $\alpha_1\beta_2$ interface (and the symmetric $\alpha_2\beta_1$) and at the C terminus of each chain. Since the $\alpha_1\beta_1$ interface (and the symmetrical $\alpha_2\beta_2$) is scarcely influenced, if at all, by O_2 binding (Perutz *et al*., 1970; Baldwin and Chothia, 1979), it is usually referred to as the frame of reference with respect to which the quaternary conformational changes are described.

At the $\alpha_1\beta_2$ interface some interactions are possible in the *T* but not in the *R* state and *vice versa*, due to the strain of the FG corner that accompanies ligand binding. As a consequence, the $\alpha_1\beta_2$ interface is so constructed to act as a two-way conformational switch (Perutz, 1970, 1998; Baldwin and Chothia, 1979), and responds to the stepwise oxygenation of the four subunits with the catastrophic all or none quaternary transition. In particular, at the α_1C-β_2FG contact, the hydrogen bond between Tyrosine α_1C7 and Aspartate β_2G1 characterizes ^{T}Hb, whereas that between Aspartate α_1G1 and Asparagine β_2G4 occurs in ^{R}Hb; moreover, in ^{T}Hb the side chain of Histidine β_2FG4 docks between Threonine α_1C6 and Proline α_1CD2, whereas in ^{R}Hb it finds its place between Threonine α_1C3 and Threonine α_1C6. The other contact region of this interface, the α_1FG-β_2C,

Table 29.2 The 'salt bridges' established by the C-terminal residues of the α and β chains in ^{T}HbA (from Perutz, 1970; Dickerson and Geis, 1983)

Carboxylate group of Arg HC3(141)α_1	with	ε amino group Lys H10 (127)α_2
Guanidinium group of Arg HC3(141)α_1	with	γ-carboxylate group of Asp H9(126)α_2
Carboxylate group of His HC3(146)β_1	with	ε-amino group of Lys C5(40)α_2
Imidazole group of His HC3(146)β_1	with	γ-carboxylate group of Asp FG1(94)β_1

accommodates the structural changes associated to ligand binding without such marked switches of the hydrogen bonding pattern and was defined the 'flexible joint' by Baldwin and Chothia (1979). The $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces remain symmetric in all published Hb structures, irrespective of ligation and allosteric conformation, and in no case it was observed that either of them is *R*-like while the other is *T*-like.

The C terminus of the α chains is constituted by the residues Tyrosine HC2 and Arginine HC3; that of the β chains by Tyrosine HC2 and Histidine HC3. In unliganded Hb the penultimate tyrosine of each chain is inserted into a hydrophobic pocket provided by helices F and H of the same chain, whereas the last residue establishes ionic interactions, in the α chain with the Valine NA1(1) of the contralateral α chain, and in the β chains with Lysine C5(40) of the contralateral α chain and with Aspartate FG1(94) of the same β chain (Perutz, 1970; see Table 19.2). Upon O_2 binding, the allosteric transition causes the expulsion of the penultimate tyrosine from its binding pocket and the breakage of the salt bridge interactions, possibly accompanied by proton release (depending on pH). Chemical or genetic alteration of the C terminal residues, or their cleavage by carboxy-peptidases, destabilizes the *T* state and greatly increases O_2 affinity.

CONTROL OF OXYGEN AFFINITY

The control of O_2 affinity has great physiological relevance and, as already stated, is exerted at tertiary and quaternary level. The main intrinsic control exerted by the globin on the reactivity of the heme iron is due to Histidine F8, which pulls the iron out of the heme plane and, in so doing, reduces its affinity for O_2 and other ligands. Consistently, mutation of Histidine F8 increases

the ligand affinity, but causes the heme to be more easily lost from the globin (Huisman *et al.*, 1996; Barrick *et al.*, 1997). Mutations or chemical modifications in the neighbourhood of F8 exert important effects on O_2 affinity without causing heme loss. An early recognized hot spot is Cysteine F9β, whose exposure to the solvent and chemical reactivity is influenced by the allosteric conformation (Perutz, 1970; Antonini and Brunori, 1971).

The stability of bound O_2 is controlled by hydrogen bond with Histidine E7, which reduces the rate of O_2 dissociation from microseconds (in the sperm whale myoglobin mutant with Valine at E7) to several milliseconds, and simultaneously increases the O_2 affinity to physiologically meaningful values; moreover, it reduces the rate of autoxidation of the heme Fe^{2+}, which otherwise would occur much too rapidly (see below). The effect of mutations of the distal Histidine (alone or in association with other residues on the distal side) on the rate constants and affinities for O_2 has been extensively investigated, mostly with sperm whale Mb as a model; proper mutations can alter the rate of O_2 dissociation from $\sim 10\,s^{-1}$ to $> 1000\,s^{-1}$, a large effect well understood from analysis of transient kinetics (Olson *et al.*, 1988).

The role of the proximal histidine extends beyond that of stabilizing bound O_2, since this residue also hinders the binding of CO, for two non-exclusive reasons: it forces an unfavorable bond angle, and it increases the polarity of the distal pocket. Indeed, in the case of O_2 the Fe–O–O bond angle is approximately 130° in model compounds and 112° in MbO_2 (Perutz, 1979), whereas CO binds with a linear geometry in model compounds, and with an angle of 120°–140° in MbCO (Kuriyan *et al.*, 1986); thus the 'natural' bond angle is more severely distorted in MbCO than in MbO_2, because of the presence of the distal His. In parallel with the distorted stereochemistry of binding, the partition coefficient between CO and O_2 is over 1000 for model compounds and only 25 for Mb.

A problem that is partially linked to that of the tertiary control of ligand affinity is the pathway for ligand entering into and escaping from the protein matrix. The X-ray structures of many hemoproteins show that the protein environment of the heme, if rigid, would prevent the entrance and the exit of even the small gaseous ligands; thus, protein fluctuations were invoked for physiological function (Ansari *et al.*, 1985). The dynamics of the heme pocket residues in sperm whale Mb, revealed by spectroscopy and crystallography, shows that Phenylalanine CD1(43) and Phenylalanine CD4(46), which wedge the prosthetic group into its pocket and are packed tightly between the heme and the distal helix E, flip over at rates faster than $10^4\,s^{-1}$; this is possible only if the entire heme pocket breathes fast. Nine clusters of residues are suggested as possible pathways for ligand escape from the interior of the protein. The most direct pathway is constituted by residues Arginine CD3(45), Histidine E7(64), Threonine E10(67), and Valine E11(68) between the CD loop, the helix E, and the heme; recent analysis of kinetic data on approx. 100 Mb mutants indicates that the ligand escape route involves by-and-large the so-called histidine gate. Our understanding of the structural dynamics of Mb has considerably increased over the last few years thanks to time-resolved Laue crystallography (e.g. Srajer *et al.*, 2001; Bourgeois *et al.*, 2003; Schotte *et al.*, 2003) and molecular dynamics simulations (Elber and Karplus, 1990).

The quaternary control of ligand affinity only occurs in Hb and acts by imposing a bias on the equilibrium between the *T* and *R* allosteric conformations. This level of control is not independent of the previous one, since the main structural factor involved is the out of plane position of the heme iron and the tension of the Fe–Histidine F8 bond that lengthens by an average of 0.2 to 0.3 Å in going from $^{R}HbO_2$ to ^{T}Hb, as a response to the movements of the F helix and FG corner that accompany the quaternary conformational change (Perutz *et al.*, 1998). Several key residues are involved in the control of the allosteric equilibrium, of which the most important are the last two residues at the C termini of both chains and those at the $\alpha_1\beta_2$ interface, as described above.

NON-HEME LIGANDS: H^+, Cl^-, CO_2 AND 2,3-DPG

The O_2 affinity of Hb is lowered by several substances that combine at sites other than the heme and are collectively identified as allosteric effectors or heterotropic ligands. Physiologically, the most important heterotropic ligands are H^+, Cl^-, CO_2 and 2,3-diphosphoglycerate (2,3-DPG). The effect of heterotropic ligands is part of the quaternary control of O_2 affinity, since they bias the allosteric equilibrium, usually in favor of the *T* state.

The modulation of O_2 affinity by H^+ is known as the Bohr effect, and reflects (1) the release of protons upon oxygenation at physiological pH (the alkaline Bohr effect), or (2) uptake of protons upon oxygenation at pH values below ~6 (the acid or reverse Bohr effect). The alkaline Bohr effect is relevant not only to O_2 transport, but also to the blood's buffer capacity. To a very crude approximation, each subunit behaves as a weak acid with pKa ~ 7.6 in deoxyHbA and pKa ~ 6.7 in oxyHbA; as a consequence of such a change of pKa, binding of one mole of O_2 causes the release of 0.5 moles of hydrogen ion (Antonini and Brunori, 1971). However, it became clear very early on that an accurate description of the Bohr effect demands more ionizable groups with subtler changes of pKa, and an obvious goal of subsequent research was the identification of the Bohr residues.

The amino-acid residues capable of binding H^+ are numerous in the protein structure, but those that effectively do so at physiological pH are only a handful; the most relevant for physiology are Valine NA1(1)α and Histidine HC3(146)β, both involved in the network of C-terminal salt bridges stabilizing ^{T}Hb. The shape and amplitude of the Bohr effect (i.e., the ratio H^+/O_2) depend strongly on the experimental conditions, and especially on the concentration of chloride ions (Bucci and Fronticelli, 1985); hence it is often difficult to compare experiments carried out in different laboratories. The α amino group of Valine NA1(1)α accounts for 25 per cent of the alkaline Bohr effect in 0.1 M Cl^-, whereas the imidazole group of Histidine HC3(146)β is responsible for 50 per cent of the alkaline Bohr effect under the same experimental conditions (Kilmartin and Rossi Bernardi, 1973; Riggs, 1988) and for almost all the Bohr effect observed in the absence of chloride. These estimates were challenged by Chien Ho, who, on the basis of NMR measurements, suggested that a larger number of residues, each experiencing much smaller pKa changes, should be considered (Ho and Russu, 1987); however, the assignment of proton resonances is difficult and these results are controversial (Riggs, 1988 and references therein).

Carbon dioxide combines with the N terminal amino groups of both chains to form a carbamino-derivative (Kilmartin and Rossi Bernardi, 1973), and decreases the O_2 affinity and the Bohr effect (Imaizumi *et al.*, 1982). Under physiological conditions Hb plays a comparatively minor role in the transport of CO_2, since over 90 per cent of this gas is physically dissolved in the blood as bicarbonate; moreover the affinity of HbA for CO_2 is relatively low, especially in view of its competition with chloride (i.e., K_d = 70 mmHg at pH = 7.4 and T = 25°C, from the data of Imaizumi *et al.*, 1982, to be compared with a physiological partial pressure of ~44 mmHg).

2,3-DPG in humans exerts the most effective control of O_2 affinity (Benesch and Benesch, 1969). It is produced by the erythrocytic enzyme glycerate bis-phosphate mutase, which uses as a reagent the glycolysis metabolite glycerate 1,3-diphosphate. 2,3-DPG binds to a specific site between the α chains and lowers the O_2 affinity of Hb by a factor of 3.5 (pH = 7.5, T = 20°C and $[Cl^-]$ = 0.1 M; data of Antonini *et al.*, 1982) by two simultaneous effects: it decreases the intrinsic O_2 affinity of *T*-state Hb and it stabilizes this allosteric state so that conversion to the R state occurs at a higher degree of O_2 saturation. 2,3-DPG binds in a cavity on the dyad axis of the tetramer, limited by the amino termini and helices H of the β subunits; it makes hydrogen bonds with Valine NA1(1), Histidine NA2(2), Lysine EF6(82), and Histidine H21(143) (Perutz, 1970). The affinity of 2,3-DPG is K_d = 3.8 mM for HbO_2 and K_d = 0.024 mM for Hb (conditions as above; data of Antonini *et al.*, 1982).

When Hb is extracted from the red cells, modified, and infused as a solution, 2,3-DPG is separated and lost in the purification procedures or because of renal filtration; as a consequence Hb solutions have a higher O_2 affinity than red cells. Several chemical modifications of Hb were devised to reduce its O_2 affinity in order to mimic the value in the erythrocyte.

Other allosteric effectors exist, and some of them are relevant to the physiology of O_2 transport. By far the most important is chloride, the concentration of which in the blood is almost constant at 0.1 M. Chloride, besides being an effector by itself, participates to the Bohr effect (see above and Bucci and Fronticelli, 1985).

NITRIC OXIDE

Nitric oxide (NO) is produced by several tissues of the human body, and acts as a bactericide and a vasodilator. It is a radical species with a single unpaired electron which reacts with Hb in different reactions (summarized in Figure 29.5), where the various possibilities outline the complexity of the chemistry (see Brunori, 2001).

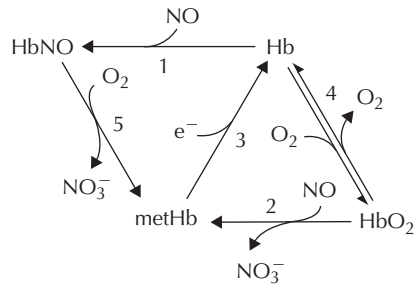


Figure 29.5 A scheme of the reactions of nitric oxide with the heme iron of Hb and Mb. The formation of a nitroso-thiol with the —SH group of Cys F9(93)β is not taken into account in this scheme. See color plate 6.

Reaction 1 in Figure 29.5 is the combination of the gas with deoxyHb and is written in a simplified form, although obviously saturation involves the cooperative binding of four molecules of NO to the tetramer. Although the affinity of NO for deoxyHb is ~200 000-folds higher than that of O_2, the reaction is also cooperative; however, the cooperativity in the binding of NO is expressed almost exclusively in the dissociation rate constants, since the combination rate constants of NO to the *T*-state and *R*-state are essentially identical (see Table 29.1). This is similar to O_2 and different from CO, as discussed in several specialized papers (Szabo, 1978). The overall rate constant for NO dissociation from ^{R}HbNO is extremely slow, and accounts for the high stability of this adduct (Moore and Gibson, 1976).

Reaction 2 in Figure 29.5 leads to oxidation of HbO_2 to metHb and formation of NO_3^-, in a quasi-irreversible very rapid second order process ($k \sim 5 \times 10^7 M^{-1}s^{-1}$; Eich *et al.*, 1996). This pathway is the most prevalent under physiological conditions, since even in the venous blood Hb is approx 70 per cent oxygenated. Quenching of free NO present in the blood is sometimes attributed to this process. Since NO is so important for the control of the blood pressure, its reactions with Hb or HbO_2 have been suggested as being responsible for the hypertensive effects observed after transfusion of Hb-based blood substitutes in laboratory animals and in patients. In the red cell, metHb formed *via* reaction 2 is reduced to functionally competent ferrous Hb by the metHb reductase enzymatic system (Surgenor, 1974).

NO also binds the metHb (Equation 29.3) to produce an iron-nitrosyl complex ($k_{on} \sim 4 \times 10^3 M^{-1} s^{-1}$ and $k_{off} \sim 1 \times 10^3 s^{-1}$). In the presence of an excess of NO, this complex undergoes reductive nitrosylation generating the reduced nitrosyl adduct and nitrite (Addison and Stephanos, 1986):

$$HbFe^{3+} + NO\,[HbFe^{3+}NO \Leftrightarrow HbFe^{2+}NO^{+}] \xrightarrow[H_2O]{NO} HbFe^{2+}NO + 2H^{+} \qquad (29.3)$$

It should be recalled that the direct reaction of NO with O_2 in solution is too slow to compete with the reactions of NO with deoxy- or oxyHb. However, alternative reactions may come into play when NO concentrations are 10^3 to 10^6 times lower than that of O_2 and Hb. In particular, when the oxygenated hemes in the quaternary R state of Hb are around 95 per cent and NO is present at even lower concentrations, the deoxy sites will bind NO, which may follow an alternative chemistry.

Over and above the reaction of NO with the heme iron, about 10 years ago it was shown that NO can react with the deprotonated sulfydryl group of Cysteine F9(93)β. Thus it was proposed that NO is transported bound to sulfydryl groups through a process called nitrosation (Jia *et al.*, 1996)

$$Hb\text{-}S^{-} + NO^{+} \leftrightarrows Hb - SNO \qquad (29.4)$$

which occurs also with glutathione, at high concentration in the erythrocytes (~2.5 mM). The reaction occurring with Cysteine F9(93)β depends on the quaternary state of Hb. According to this hypothesis, NO binds to the minor (~1 per cent) population of deoxygenated heme in oxygenated erythrocytes present in the arterial circulation, forming iron-nitrosyl-Hb; the NO group is then transferred (through a one-electron oxidation) to Cysteine F9(93)β to produce Hb–SNO (< 50 nM *in vivo*), which dissociates NO upon deoxygenation in the peripheral tissues, and elicits vasorelaxation *via* trans-nitrosation reactions to low molecular mass thiols (such as glutathione, yielding S-nitroglutathione).

This new pathway for 'storage and transport' of NO was suggested to be a key event in the control of blood pressure and vasodilatation, an hypothesis championed by Stamler and coworkers (Jia *et al.*, 1996; Pawloski and Stamler, 2002) but not totally accepted and in fact questioned by others. Dissent mostly concerns the wide spectrum of analytical approaches employed to measure NO and nitrosothiols, which often yield divergent values, leading to conclusions

with many aspects disputed. In particular, the main dissent rests on the similarity or difference in levels of S-nitrosothiols and $HbFe^{2+}NO$ between arterial and venous blood: thus, in contrast to the proponents of the model, who assign an essential physiological role to Hb–SNO transport, opponents report no artery-to-vein gradient (Gladwin *et al*. 2003).

AUTOXIDATION

HbO_2 undergoes spontaneous oxidation to the aquo-met or hydroxy-met derivative, with release of O_2^- (Misra and Fridovich, 1972). Inside the red blood cell an enzymatic system reduces metHb and maintains its fraction at about 2–3 per cent, without adverse physiological effects. The reaction mechanism is consistent with the proposal that the oxygenated derivative has the partial character of a Fe^{3+}–O_2^- complex (Weiss, 1964; Antonini and Brunori, 1971):

$$[Fe^{2+} - O_2 \leftrightarrow Fe^{3+} - O_2^-] \rightarrow Fe^{3+} + O_2^- \quad (29.5)$$

Autoxidation, however, is more complex, and involves at least another path, which was elucidated by the combined effort of several authors (Antonini and Brunori, 1971; Wallace *et al*., 1982; Brantley *et al*., 1993). A crucial observation was that the autoxidation rate exhibits a bell-shaped dependence on O_2 partial pressure, being maximal at partial saturations (0.25 to 0.95) and declining at higher and lower PO_2. This result demands that deoxyHb is an intermediate in the oxidation, most probably through the transient binding of Cl^- or H_2O (Wallace *et al*., 1982; Yusa and Shikama, 1987; Brantley *et al*., 1993). This second reaction path is described by:

$$Fe^{2+} - O_2 + X \rightarrow Fe^{2+} - X + O_2 \rightarrow Fe^{3+} - X + O_2^- \quad (29.6)$$

which suggests that the five-coordinated Fe^{2+} and the six-coordinated Fe^{2+}–O_2 resist oxidation by the external oxidant (O_2), but the transiently formed six-coordinated species Fe^{2+}–X oxidizes readily. The hydrophobic distal pocket helps to keep the polar species X far from the iron but allows the entrance of gases which, once bound, prevent liganded Hb from entering reaction path 29.7.

A study on the role of the distal Histidine E7 has shown that two factors contribute to reduce the autoxidation rate constant of Mb: the high O_2 affinity (which reduces the fraction of unliganded Mb) and the low polarity of the pocket (which prevents the entry of anions). Site directed mutagenesis on Mb demonstrated that increasing the polarity of the heme pocket (e.g. by replacement of Valine E11 with Threonine or Serine) markedly increases the autoxidation rate, while decreasing the volume of the heme pocket (e.g. by substitution of Valine E11 with Leucine or Phenylalanine, or of Leucine B10 with Phenylalanine) decreases this rate (Brantley *et al*., 1993). Very high autoxidation rates are also observed in mutants bearing Tyrosine at E7 or F8, that directly coordinates the heme iron, or anionic residues in the heme pocket. This occurs naturally in the pathological Hbs called methemoglobins (HbM Boston: Histidine αE7 → Tyrosine; HbM Iwate: Histidine αF8 → Tyrosine; HbM Saskatoon: Histidine βE7 →Tyrosine; HbM Hyde Park: Histidine βF8 → Tyrosine; HbM Milwaukee I: Valine βE11 → glutamate; see Huysman *et al*., 1996).

The autoxidation rate constants of the α and β chains of HbA at physiological pH differ by a factor of 10 in the tetramer and by a factor of 25 in the isolated state; however, this nonequivalence vanishes at pH 9.0. The rate constants determined for tetrameric HbA are $k_\alpha = 0.032\,h^{-1}$; $k_\beta = 0.0037\,h^{-1}$ at 37°C and pH 7.2 (Mansouri and Winterhalter 1973).

EDITOR'S SUMMARY

No protein has been studied in more detail than hemoglobin, and the presence of hemoglobin in the circulation is critical for the existence of all higher organisms, including humans. A tetrameric protein, hemoglobin contains four heme groups, each of which contains an iron atom that binds O_2 reversibly. The molecular mechanisms that underlie this O_2 binding can explain the control of O_2 affinity, cooperativity (the sigmoid oxygen equilibrium curve) and the influence of the allosteric effectors H^+, Cl^-, 2,3-DPG and CO_2. These structural mechanisms are intimately related to the interaction of the hemoglobin subunits, controlled by the amino

acid residues that make up the interfaces between subunits. At low concentrations, such as would be found outside the red blood cell, these same subunit interactions would allow the tetramers to dissociate into dimers, which would then be rapidly removed by the kidney and could also enter the extravascular spaces.

Within the red blood cell, efficient enzyme systems ensure that the iron atom remains in the reduced (Fe^{2+}) state, a requirement for binding O_2. Without such mechanisms iron would oxidize to Fe^{3+}, releasing an electron. Ferric hemoglobin does not bind O_2 and may catalyze toxic reactions. It is now known that NO, which binds to hemoglobin with great avidity, is also a local vasodilator. Outside the red cell, hemoglobin can very efficiently scavenge NO.

The challenge to develop a 'blood substitute' based on hemoglobin, regardless of its source, is to control subunit dissociation, prevent oxidation and vasoconstriction, prevent disappearance from the circulation, and ensure that it can deliver O_2 to tissue. Due to the linkage of structure and function of this unique and complex protein, this has been a daunting undertaking.

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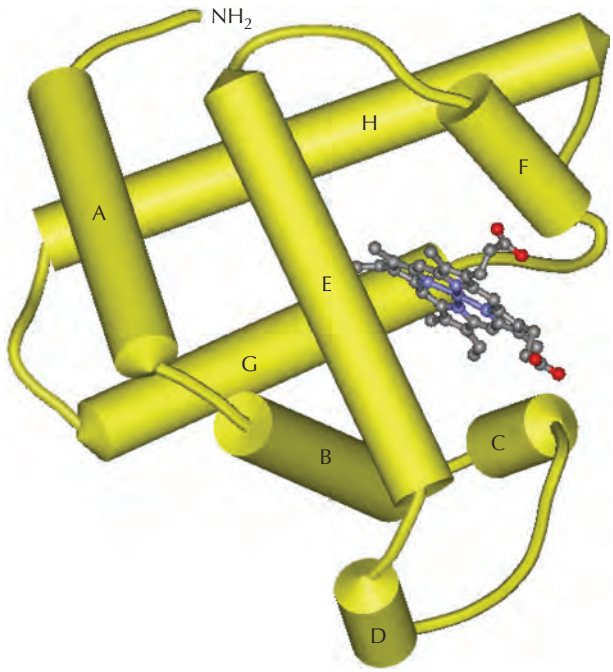

Plate 5 The 'globin fold' as exemplified by sperm whale myoglobin. The eight α-helices are named A through H. See Fig. 29.1.

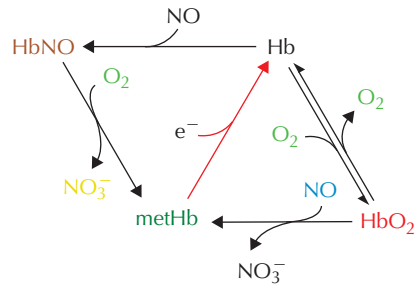

Plate 6 A scheme of the reactions of nitric oxide with the heme iron of Hb and Mb. The formation of a nitroso-thiol with the —SH group of Cys F9(93)β is not taken into account in this scheme. See Fig. 29.5.

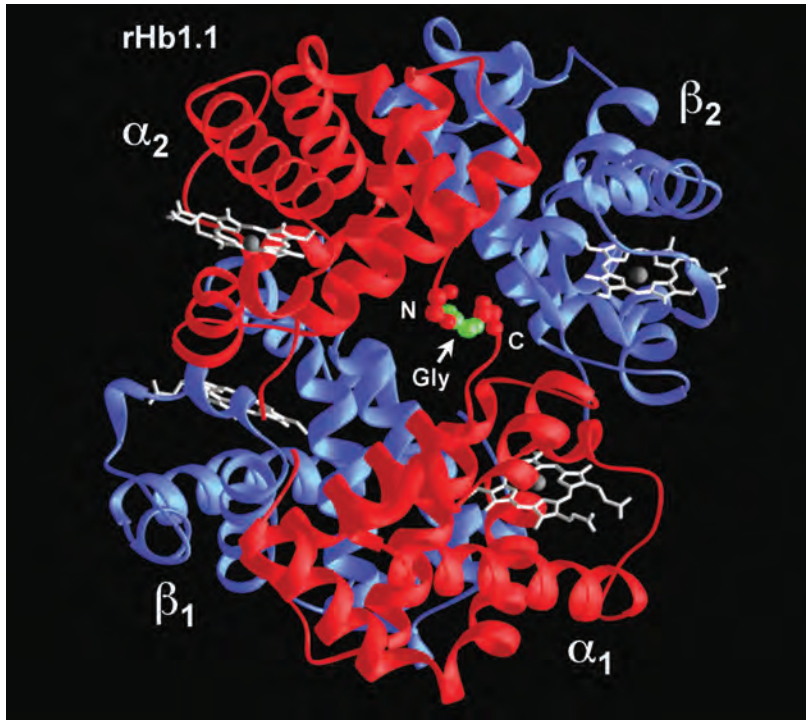

Plate 7 Structure of deoxy rHb1.1 showing the single glycine linker between the C-terminus of α_1 and the N terminus of α_2 (PDB accession code 1C7C (Brucker, 2000). See Fig. 31.1.

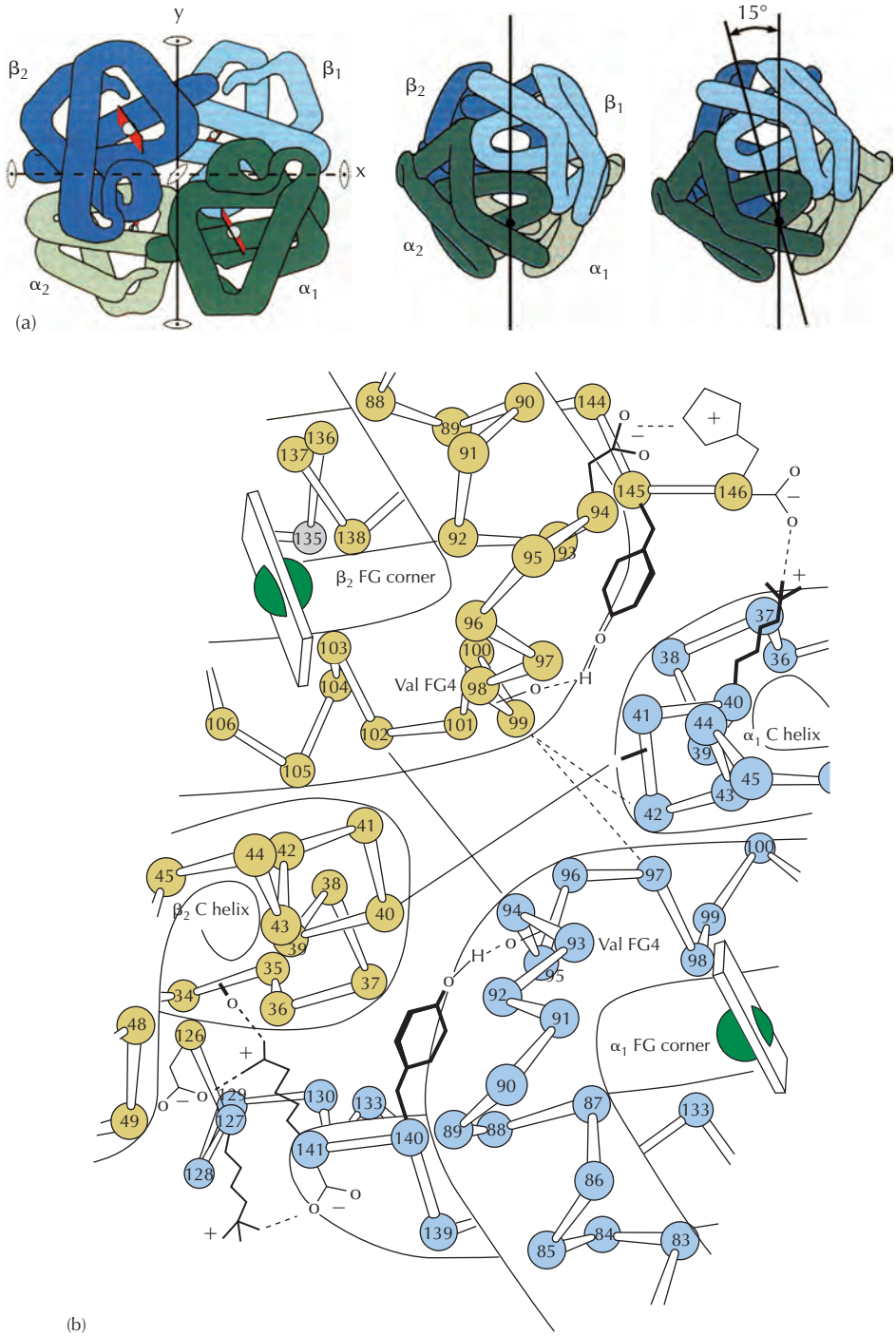

Plate 4 The allosteric transition in HbA. (a) Overview of the allosteric transition (reproduced with permission from Eaton *et al.*, 1999). The allosteric transition consists mainly of a sliding and a rotating motion of the $\alpha_1\beta_1$ dimer with respect to the $\alpha_2\beta_2$, with a consequent rearrangement of the $\alpha_1\beta_2$ interface and the symmetric $\alpha_2\beta_1$. (b) Details of the amino acid contacts at the $\alpha_1\beta_2$ interface (modified after Dickerson and Geis, 1983). See Fig. 29.2.

Chapter 30

Hemoglobin Modification

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INTRODUCTION

Joseph Barcroft, as early as 1922, expressed the view that hemoglobin must be packaged inside the red cell because otherwise its viscosity and colloid osmotic pressure would be so high as to cause deranged function (Barcroft *et al.*, 1923). Amberson (1937) agreed and extended this view:

> We fully agree, however, with Barcroft's argument that the red cell exists to prevent escape of hemoglobin into urine and lymph and to furnish a chemical environment in which hemoglobin may function with maximal efficiency, to which we would add the fact that a major factor in this chemical control is to maintain hemoglobin in its reduced state, electrochemically speaking, so that it is not transformed into physiologically useless methemoglobin.

The problems associated with the use of unmodified, cell-free hemoglobin as a red cell substitute are that, although hemoglobin has the desirable properties of a high capacity to bind oxygen and to release it cooperatively, which makes it attractive for use as a red cell substitute, when it is free in solution it has several unique properties:

1. Its oxygen affinity is high because outside of the red cell the normal allosteric effector, 2,3-diphosphoglycerate (2,3-DPG), is not present
2. Its effectiveness as an oxygen carrier is limited because it dissociates into half-molecules ($\alpha\beta$ dimers) that are rapidly removed from the circulation by the kidney after filtration in the glomerulus
3. Once filtered, a high concentration of protein in the renal tubules can cause tubular obstruction and consequent renal failure

Thus, to be an effective oxygen carrier in the cell-free state, hemoglobin must be chemically modified to avoid these problems. Some authors have felt that the high colloid osmotic pressure (COP) of cell-free hemoglobin is another problem that needs to be addressed by modification, but as yet there is no agreement regarding the optimal COP of a plasma expander (see Chapter 10).

The goals of chemical modification are to decrease the oxygen affinity of hemoglobin and to prevent its dissociation into $\alpha\beta$ dimers. Secondary goals are to perform such modifications with a high yield of product and to obtain derivatives of such purity that specific biologic and chemical properties can be correlated and toxicologic studies can be carried out and interpreted.

Numerous studies of the biologic and toxicologic effects of infusions of hemoglobin solutions have been carried out over the past century, many in the hope of developing a hemoglobin-based blood substitute. At this time there is still no product available for clinical use, and an enormous

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confusing and conflicting literature has accumulated regarding its 'toxic' effects. Still, there is no clear demonstration that hemoglobin or its derivatives are toxic. The goal of this chapter is to review some of the current strategies of chemical modification and to try to show some of the reasons why purity of products is both difficult to achieve and of the highest importance. No attempt will be made to present an exhaustive catalogue of all of the chemical modifications of hemoglobin which have been attempted.

REACTIVITY OF HEMOGLOBIN

Hemoglobin can exist in either of two structural conformations, corresponding to the oxy (*R*, relaxed) or deoxy (*T*, tense) states. The key differences between these two structures are that the constrained *T* state has a much lower oxygen affinity than the *R* state and the *T* state has a lower tendency to dissociate into subunits that can be filtered in the kidneys. Therefore, stabilization of the *T* conformation would be expected to both reduce renal filtration and maintain oxygen affinity similar to that of red cells. The transition between the *T* and *R* states of hemoglobin is also critical for the Bohr (pH) effect and cooperativity. Therefore stabilization of either of the two structures should diminish these effects, which might have important physiologic consequences.

Stabilization of the *T* conformation under normal conditions is illustrated by the reaction of 2,3-diphosphoglycerate (2,3-DPG) (Figure 30.1). The negative charges on this polyphosphate form electrostatic, reversible interactions with eight positive charges on hemoglobin: two α-amino groups of valine NA1(1)α, two ε-amino groups of lysine EF6(82)β and four histidines, NA2(2)β and HC3(143)β. In the *R* state the dimensions of the pocket change enough so that 2,3-DPG does not fit as well, and it drops out. Thus 2,3-DPG preferentially stabilizes the *T* conformation and has an overall effect of reducing oxygen affinity and increasing cooperativity. Analog of 2,3-DPG, used to modify hemoglobin by forming permanent covalent bonds, are variously effective, depending on molecular dimensions and charge. Some of the compounds bind to only one of the reactive amino groups in the 2,3-DPG pocket; others react with all four.

In addition to the 2,3-DPG pocket, human hemoglobin contains 40 reactive lysines, i.e., ε-amino groups, two α-chain N-terminal α-amino groups, and two sulfhydryl groups, i.e., cysteine

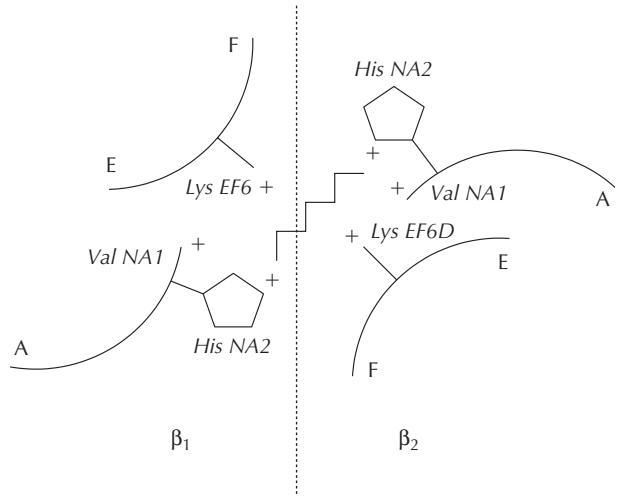

Figure 30.1 Reaction of diphosphoglycerate (2,3-DPG) and deoxyhemoglobin. The molecule fits into the central cavity of hemoglobin and forms salt bridges with valine NA1(1)β, histidines NA2(2)β, H121(143)β and lysine EF6(82)β. A, E, and F refer to specific hemoglobin helices and NA is the sequence from the amino-terminals to the A helix.

F9(93)β. Most of the lysines are on the surface of the molecule, but some are internal, such as lysine G6(99)α. Thus the groups can be accessed by various crosslinkers and polymerizing agents, especially aldehydes. Although the lysine groups provide many potential sites for modification, their large number also means that such reactions are difficult to control.

All of the reactions considered to be useful in the production of hemoglobin-based blood substitutes use chemical modification at one or more of the sites discussed above. Table 30.1 lists the different types of modifications with examples of the most common reactions for each. Differences in the reactions are determined by the dimensions and reactivity of the crosslinking reagents. Because the function of hemoglobin in binding and releasing oxygen is intricately connected to the transition between *T* and *R* conformations, it is not surprising that *P*50 and yield are highly variable. Even small differences among structures of the reagents can yield products having very different properties. In addition, the conditions of the reaction are very important, not only in regard to the state of ligation, i.e., oxygen saturation, but also in regard to the presence of agents or molecules that block or compete for certain reactive sites.

A further complication of these reactions is that many non-hemoglobin proteins also contain reactive groups which may be co-modified. These molecules, if present at the time of reaction, could

Table 30.1 Classes of hemoglobin modification

Class	Examples
Amino-terminal modification	Carbamylation Carboxymethylation Pyridoxylation Acetaldehyde
Lysine EF6(82)β modification	Mono(3,5-dibromosalicyl)-fumarate
Valine NA1(1)β-Lysine EF6(82)β crosslink	2-nor-2-formylpyridoxal 5′-phosphate (NFPLP) Bis-pyridoxal tetra-phosphate (bis-PL)P_4
Lysine G6(99)α_1-Lysine G6(99)α_2 crosslink	Bis(3,5-dibromosalicyl)-fumarate
2,3-DPG analog	Pyridoxal 5′-phosphate
Surface, multisite	Glutaraldehyde Polyaldehydes Ring-open dials Diimidate esters
Conjugated hemoglobin	Dextran, starch aldehydes Poly(ethylene glycol)

affect the properties of the final solution. For this reason, derivatives prepared for studies of the hemoglobin molecule *per se* must start with highly purified stroma-free hemoglobin.

AMINO-TERMINAL MODIFICATION

Carbamylation

Modification of the amino-terminal groups of hemoglobin (Figure 30.2) by the carbamylation reaction using isocyanic acid was used to show that Valine NA1(1)α is one of the residues involved in the alkaline Bohr effect, and that the sickling of cells containing hemoglobin S could be inhibited specifically (Manning, 1981). It was also used to show the site of binding of carbon dioxide. The products of these reactions have increased oxygen affinity if the α-chains are carbamylated and decreased affinity if the β-chains are carbamylated. The carbamylation reaction held great promise in the control of sickling, but orally administered sodium cyanate was toxic.

Carboxymethylation

Other modifications of the amino-terminal groups of hemoglobin have also been studied. It was reasoned that a covalent adduct at the amino-terminal amino group might reduce oxygen affinity by lowering or neutralizing the net positive charge in the 2,3-DPG pocket (Fantl *et al.*, 1987a). Carboxymethylation using glyoxylic acid followed by reduction with sodium borohydride, $NaBH_4$, resulted in a product that demonstrated lowered oxygen affinity and nearly intact Bohr and carbon dioxide effects (Manning *et al.*, 1989). X-ray and nuclear magnetic resonance studies confirmed that the introduced group occupies nearly the same position as the naturally occurring carbamino group, i.e., carbon dioxide adduct (Fantl *et al.*, 1987b).

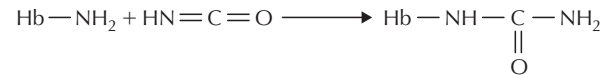

Figure 30.2 Carbamylation of the α-amino groups of hemoglobin.

Acetaldehyde

Acetaldehyde, C_2H_4O, has a slightly different reaction mechanism with hemoglobin (San George and Hoberman, 1986). Although this reagent reacts with surface lysines under some conditions, the principal products are derivatives of the amino-terminal groups, both of the αH and β-chains. These products are not reduced with sodium borohydride and therefore do not involve an intermediate Schiff's base. Instead, a stable cyclic imidazolidinone derivative is formed.

MODIFICATION AT THE 2,3-DPG BINDING SITE

Pyridoxal derivatives

Various aldehydes of pyridoxal react with hemoglobin at sites that can be somewhat controlled by the state of oxygenation (Benesch and Benesch, 1981). It is thereby possible to prepare derivatives having a wide range of functional properties. The reaction of PLP with hemoglobin involves first the formation of a Schiff's base between the amino groups of hemoglobin and the aldehyde(s) of the pyridoxal compound, followed by reduction of the Schiff's base with sodium borohydride to yield a covalently-linked pyridoxyl derivative in the form of a secondary amine. This reaction has been used widely to

reduce the oxygen affinity of the final product (Benesch and Benesch, 1981).

Pure diPLP-hemoglobin, in which both β-chain amino termini are modified, was isolated by column chromatography, and the structure was confirmed by X-ray diffraction (Arnone *et al.*, 1977) and peptide analysis (Benesch *et al.*, 1982). An electrostatic interaction of the 5′-phosphate with the 2,3-DPG binding site, lysine EF6(82)β, was shown, so that this modification closely mimics the action of 2,3-DPG in stabilizing the deoxy conformation. The oxygen affinity of the derivative was found to be about half that of unmodified hemoglobin under similar conditions, but a degree of cooperativity was preserved. Equilibrium and kinetic ligand-binding studies on this derivative (Bellelli and Brunori 1986) showed a perturbed *R* state.

The reaction of hemoglobin with PLP was scaled up (Sehgal *et al.*, 1981; DeVenuto and Zegna, 1983) to batches of 20 l yielding 70–80 per cent modified hemoglobin. Methemoglobin was less than 10 per cent, and the material was apparently unchanged after infusion into baboons. This solution was effective in resuscitation from hemorrhagic shock (Hoyt *et al.*, 1980; Jesch *et al.*, 1982), but the plasma retention was thought to be too short and colloid osmotic pressure (COP) too high to be a definitive red cell substitute (Sehgal *et al.*, 1984; Gould *et al.*, 1986; Moss *et al.*, 1988). A major problem with the large-scale preparation of pyridoxylated hemoglobin was the heterogeneity of reaction products, probably representing modifications at either or both α and β amino-terminal residues as well as surface lysines.

lysine EF6(82)β modification

In this reaction, sometimes called a 'pseudolink' (Bucci *et al.*, 1989a) hemoglobin reacts with the monofunctional reagent, mono(3,5-dibromosalicyl)fumarate, in oxygenated conditions. The product is specifically acylated at lysine EF6(82)β, in about 70 per cent yield. Although cooperativity is reduced somewhat, i.e., to a Hill coefficient of 2.0, the *P*50 under physiologic conditions is about 25 mmHg, and carbon dioxide binding is intact, because the sites for carbon dioxide binding are unaffected. It is of particular interest that the tetramer–dimer dissociation is retarded, possibly by stabilization at the β–β interface (Bucci *et al.*, 1989b). The resulting plasma retention half-time in the rat is also prolonged by about four-fold for this acylated material, as compared to unmodified hemoglobin.

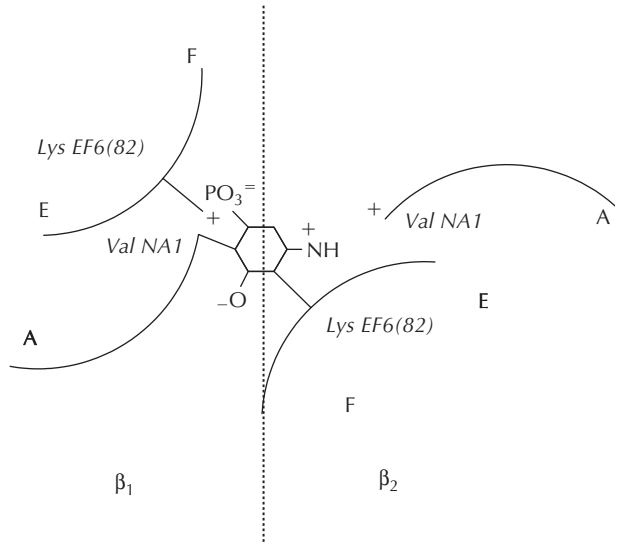

Figure 30.3 Reaction of 2-nor-2-formyl pyridoxal 5′-phosphate (NFPLP) and hemoglobin. A, E and F refer to helices; NA is the sequence from the amino-terminals to segments A.

2-Nor-2-formylpyridoxal 5′-phosphate

2-Nor-2-formylpyridoxal 5′-phosphate (NFPLP) is of special interest because it contains two reactive aldehyde groups and reacts as shown in Figure 30.3 at two sites: at the amino-terminal group of one β-chain and at lysine 82 of the other (Benesch *et al.*, 1975; Arnone *et al.*, 1977). Thus in one modification reaction this reagent both reduces the oxygen affinity of native hemoglobin and prevents its dissociation into αβ-dimers.

NFPLP has been studied extensively. Because hemoglobin dimerization is prevented, NFPLP is not eliminated in the urine (Sloviter *et al.*, 1981; Triner *et al.*, 1983; Keipert *et al.*, 1988) and the plasma retention of the modified material is at least three times that of either unmodified hemoglobin or pyridoxylated hemoglobin (VanderPlas *et al.*, 1986). Tissue distribution and elimination have been documented in detail (Ayer and Gauld, 1942; Bleeker *et al.*, 1989a; Keipert and Triner, 1989). Accumulation of this modified hemoglobin derivative in the kidney is much reduced as compared to unmodified hemoglobin (Bleeker *et al.*, 1989b), and the oxygen affinity of the derivative under physiologic conditions is about 47 mmHg, with cooperativity retained. When used to perfuse isolated organs, the derivative supports a higher tissue oxygen tension in both the rabbit heart

(Benesch *et al.*, 1984) and the rat liver (Bakker *et al.*, 1986).

Although the 60–80 per cent yield of the NFPLP product may have been satisfactory for commercialization (VanderPlas *et al.*, 1987), the main drawback was difficulty in preparation of the reagent itself.

A second class of bifunctional reagents, described in 1988, involves two pyridoxal groups linked by phosphates of different lengths (Benesch and Kwong, 1988). The yield of intramolecularly crosslinked hemoglobin increases dramatically with increasing length of the phosphate backbone. It is believed that the site of reaction of (bis-PL)P_4 is between the amino-terminal amino group of one β-chain and the lysine 82 of the other β-chain, as for NFLP (Benesch and Kwong, 1988). However, the distance between these two residues is only 1.1 nm, and the reagent is much longer. Therefore it is concluded that the crosslinker must fold back upon itself to form a stacked pyridine ring conformation.

Further study of (bis-PL)P_4 modified hemoglobin (Keipert *et al.*, 1989) showed its *P*50 to be 31 mmHg (pH 7.4, PCO_2 40 mmHg, 37°C), with a Bohr effect about half that of unmodified hemoglobin. Its plasma retention is prolonged threefold in the rat, and there was no apparent toxicity in screening studies. An attractive feature of (bis-PL)P_4 is that its synthesis is much simpler than that of NFPLP-hemoglobin (Winslow, 1989).

Other 2,3-DPG pocket crosslinkers

The reactivity of the valine NA1(I)α and lysine EF6(82)β residues in the 2,3-DPG pocket shown by NFPLP and (bis-PL)P_4 stimulated the search for other reagents that react similarly but have potentially greater efficiency and ease of scale-up. The systematic study of four different dicarboxylic acid derivatives, crosslinked in both oxygenated and deoxygenated conditions, has been reported (Jones *et al.*, 1989). Each of these derivatives presents problems in purification, and proof of the sites of reaction is tedious.

αα-CROSSLINK (LYSINE G6(99) α_1–LYSINE G6(99)α_2)

A class of bifunctional reagents that crosslink human hemoglobin internally to preserve the native dimensions of the molecule has been very useful in the production of a well characterized

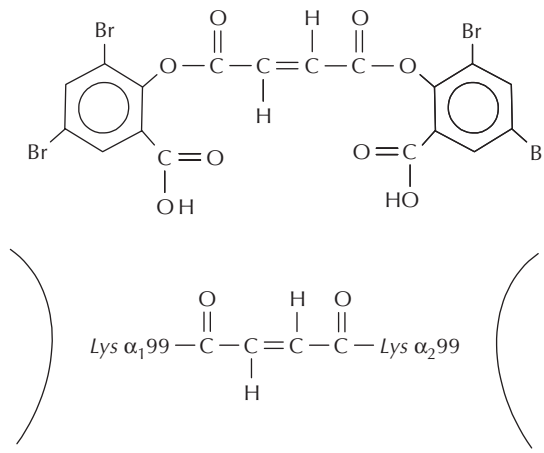

Figure 30.4 Structures of bis(3,5-dibromosalicul) fumarate (DBBF) and the αα-hemoglobin crosslink (150).

product for research purposes (Zaugg *et al.*, 1975; Walder *et al.*, 1979, 1980; Wood *et al.*, 1981). The derivatives increased the oxygen affinity of native hemoglobin and were thought to have potential in preventing sickling in patients having sickle-cell disease. When oxyhemoglobin was crosslinked using bis(3,5-dibromosalicyl)fumarate (DBBF), the reaction site was shown to be between lysine EF6(82)β_1 and lysine EF6(82)β_2 (Walder *et al.*, 1980). However, when crosslinking was carried out in deoxyhemoglobin, the α-chains were modified (Walder *et al.*, 1982; Snyder *et al.*, 1987; Figure 30.4).

This hemoglobin derivative was proposed to be developed as a blood substitute (Tye *et al.*, 1983; Snyder *et al.*, 1987) because a single modification could achieve the dual goals of reduced oxygen affinity and restricted tetramer–dimer dissociation. The product, called αα-Hb by the US Army and DCLHb by Baxter, was formulated in Ringer's acetate by the Army. *P*50 under physiologic conditions was approximately that of human blood (MacDonald and Winslow, 1992), Hill's parameter was 2.2, and the Bohr effect was reduced (Vandegriff *et al.*, 1989). Plasma retention was increased, and the product appeared to be less heterogeneous than some of the other derivatives under study. An interesting property of αα-Hb is its thermal stability, which was exploited to achieve both a partial purification of the crude reaction mixture after crosslinking and inactivation of viruses in the final product (Estep *et al.*, 1988, 1989). Its production was scaled up by Baxter Healthcare, under contract to the US

Army, but later abandoned because of its propensity to cause vasoconstriction in animals and man (Winslow, 2000).

SURFACE, MULTISITE POLYMERIZING REAGENTS

Surface modification of hemoglobin with multifunctional aldehydes has been one of the most popular modifications because it results in large aggregates of molecules with potentially prolonged intravascular retention time. An inherent problem is that the extent of polymerization may be both non-specific and difficult to control. Glutaraldehyde is a prime example of such a reagent (Figure 30.5).

Glutaraldehyde

Polymerization of pyridoxylated human hemoglobin using glutaraldehyde was first reported in 1980 (Sehgal *et al.*, 1980). In the years that followed, this polyhemoglobin was studied intensively, and a research team at Michael Reese Hospital in Chicago produced a glutaraldehyde-polymerized human hemoglobin product that has been tested extensively in humans (Gould *et al.*, 2002).

The production process begins with pyridoxylated hemoglobin (12–14 g/dl) that is then polymerized using a 12.5 per cent solution of glutaraldehyde. When the colloid osmotic pressure (COP) of the reaction mixture reaches normal values of 20–25 mmHg, the reaction is quenched by the addition of an amino acid such as 1.3 M lysine. The resulting product has a distribution of molecular masses from 64 to 400 kDa, a *P*50 of about 18mmHg, a Bohr effect reduced by half, and a Hill coefficient of 1.7. The viscosity is about 2 cPs (compared to 4 cPs for human blood) and the solution has no effect on coagulation, as measured by the prothrombin and partial thromboplastin times. Some of the properties of glutaraldehyde hemoglobin are shown in Table 30.2. Physiologic studies with a glutaraldehyde derivative with molecular weight of 124 kDa (DeVenuto and Zegna, 1981) showed that the product transports oxygen as expected and that the reduced *P*50 did not diminish its usefulness (Rosen *et al.*, 1986). The plasma half-life in baboons was up to 46 hours, compared with about 6 hours for PLP-hemoglobin (Sehgal *et al.*, 1984).

Re-examination of the products of the glutaraldehyde reaction of pyridoxylated hemoglobin revealed extreme heterogeneity (Agostoni *et al.*, 1987; Marini *et al.*, 1989, 1990) and showed that the products are unstable on storage at 4°C; rearrangements of polymeric species occur so that it is difficult to prepare a predictably modified species. This heterogeneity and instability are regarded as serious drawbacks to the product because reactions with plasma proteins *in vivo* would be impossible to predict and toxicity difficult to understand. Concern has been raised (Agostoni *et al.*, 1987) that the low molecular weight material might be preferentially lost through the kidneys, leaving the inherently less stable polymers with the less favorable oxygen transport properties and vasoactivity.

Glutaraldehyde treatment of hemoglobin has the effect of making the tetrameric structure of the molecule more rigid. Indeed, it seems that the more highly modified the polymerized hemoglobin molecules are, the more rigid they become, as reflected by increasing oxygen affinity and decreasing cooperativity. Studies using the very sensitive Mossbaur technique (Guillochon *et al.*, 1986) have shown that glutaraldehyde-treated hemoglobin has an increased rate of autoxidation and increased thermal stability. These properties could be explained by a weakening of the heme–globin linkage.

Toxicology studies with glutaraldehyde products are of great concern because glutaraldehyde can leach out of prosthetic devices (Gendler *et al.*, 1984; McPherson *et al.*, 1986). Glutaraldehyde is also used as a tissue fixative, and even small

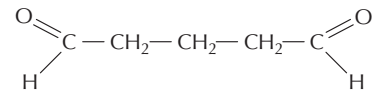

Figure 30.5 Glutaraldehyde.

Table 30.2 Properties of a typical glutaraldehyde polymerized hemoglobin product

Hemoglobin concentration, g/dl	12 to 14
Methemoglobin (%)	<5
Molecular weight (kDa)	64 to 400
Mean molecular mass (kDa)	150
*P*50 (Torr)	~18
n (Hill parameter)	1.5 to 2.2
Bohr factor (Dp50/DpH)	−0.12 to −0.25
Colloid osmotic pressure, mmHg	~25
Viscosity (cPs)	~2
Endotoxin (EU/ml)	<0.6
Rabbit pyrogen test	Pass

amounts have been found to have cytotoxic activity (Speer *et al.*, 1980). In spite of these concerns, a glutaraldehyde-polymerized product has successfully been tested in humans, and at the time of this writing is undergoing a Phase III study in pre-hospital trauma (see Chapter 13). A glutaraldehyde-polymerized bovine hemoglobin has also been produced in large scale, and is currently in human trials (see Chapters 36 and 37).

Other polyaldehydes

Other dialdehyde reagents can be prepared by oxidizing the ring structures of sugars or nucleotides (Scannon, 1982). These reagents can react with hemoglobin at any of its amino groups and therefore form a variety of modifications, including intramolecular and intermolecular links. One example of this type of modification involves opening the ring of inositol tetraphosphate. Another example involves the opening of the pyridine ring of ATP (Greenburg and Maffuid, 1983) to form modified ATP-hemoglobin. This latter product was reported to have an elevated *P*50 and normal cooperativity.

Optimization of the ATP-hemoglobin reaction conditions produced a derivative with a reduced oxygen affinity. Five fractions from a reaction mixture, when isolated, were found to have *P*50 values ranging from 8 to 38 mmHg, most with little cooperativity (McGarrity *et al.*, 1987). These results are consistent with those found with other polyfunctional reagents that react on the surface of hemoglobin.

A product based on human hemoglobin, polymerized with ring-opened raffinose, has been extensively studied and developed as far as Phase II clinical trials (Adamson *et al.*, 1998; Lieberthal *et al.*, 1999; Carmichael *et al.*, 2000; Hill *et al.*, 2002). Recently, testing was halted because of the finding of cardiac toxicity.

Diimidate esters

Diimidate esters are bifunctional reagents that have been used in crosslinking a variety of proteins including hemoglobin. In a typical reaction, a lysyl ε-amino group reacts with the ester. The reagent is specific for surface ε-amino groups and forms polymers of varying size. One of the advantages of the reaction is that it replaces the $—NH_3^+$ group with an $=NH_2^+$ group, so the overall charge is unchanged. One reported product (Mok *et al.*, 1975) had 30 of the 44 surface Lysyl residues modified and had a molecular mass ranging from 68 to 600 kDa. Intravascular retention time was increased by about four-fold in rabbits.

'Zero-link' polymers

Zero-link polymerization is a variety of polymerization developed by researchers at the University of Maryland (Matheson *et al.*, 2002; see Chapter 42). This product is called 'zero-linked' hemoglobin (ZL-HbBv) because the chemistry involves direct coupling of hemoglobin molecules together without using polymerizing agents such as glutaraldehyde or other bifunctional agents. The key property of ZL-HbBv appears to be its very large molecular size, which results in reduced extravasation compared to native hemoglobin. The developers of ZL-HbBv believe that this reduced extravasation avoids nitric oxide binding, which would cause vasoconstriction, on the theory that hemoglobin in the interstitial space more effectively binds NO than does intravascular hemoglobin.

CONJUGATED HEMOGLOBIN

An alternative approach to prolongation of the plasma retention is to conjugate hemoglobin to a larger molecule. This was first done by coupling hemoglobin to dextran (Tam *et al.*, 1976; Chang and Wong, 1977; Blumenstein *et al.*, 1978). The coupling reaction is carried out using a lysate of human red cells and bromodextran, molecular weight 20 kDa. The product was shown to support life in the absence of red cells in dogs and cats (Humphries *et al.*, 1981), and it did not appear to be immunogenic (Cunnington *et al.*, 1981). Because the oxygen affinity of dextran-hemoglobin was essentially that of hemoglobin, it was modified further by covalently linking an analog of inositol hexaphosphate (IHP) (Wong, 1988; Wu *et al.*, 1989). This new derivative had a *P*50 of 55 mmHg (compared to 23 mmHg) for dextran-hemoglobin, and the oxygenation curve showed cooperativity. These modifications were demonstrated to reduce renal toxicity of unpurified hemoglobin.

Hemoglobin also can be conjugated to other synthetic polymers such as inulin (Iwasaki *et al.*, 1983), poly(vinylpyrrolidinone) (Schmidt, 1979), and poly(ethylene glycol) (PEG) (Ajisaka and Iwashita, 1980; Leonard and Dellacherie, 1984).

One of the most studied of these conjugates is pyridoxylated hemoglobin- polyoxyethylene (PHP) (Ajisaka and Iwashita, 1980). PHP has a molecular mass of about 90 kDa and a *P*50 of about 22 mmHg, compared to 15 mmHg for hemoglobin under the same conditions, but it has reduced cooperativity (Matsushita *et al.*, 1988a). The plasma retention half-life in dogs is about 36 hours, and it apparently causes no renal, hepatic or coagulation toxicity. Histologic examination of the lungs and spleen of transfused dogs at 2 weeks and later showed no abnormalities (Matsushita *et al.*, 1988b).

A new conjugated hemoglobin product is based on a novel view of the physiology governing microvascular blood flow. According to this theory, microvascular vasoconstriction in the case of cell-free hemoglobin does not result entirely from NO scavenging, but from oversupply of O_2 to regulatory arterioles because of facilitated diffusion of hemoglobin in the plasma space (McCarthy, 2001). The product (MP4, see Chapter 40) controls facilitated diffusion by its large molecular size and high O_2 affinity. It is produced by novel attachment of six strands of maleimide-poly(ethylene glycol) (5 kDa) to human hemoglobin in a site-specific reaction (Vandegriff *et al.*, 2003). MP4 has been shown to be free of vasoconstriction in the hamster microcirculation and does not elevate systemic or pulmonary vascular resistance in pigs, nor does it cause myocardial lesions in rhesus monkeys. The chemistry involves the surface modification of ε-amino groups of lysine followed with site-specific coupling of maleimide-activated PEG (Acharya *et al.*, 1996). The product is extremely homogeneous.

RECOMBINANT HEMOGLOBIN

Human hemoglobin has been expressed in a variety of recombinant systems, including yeast and transgenic animals, but in large scale in *E. coli* (Hoffman *et al.*, 1990; Looker *et al.*, 1992). A novel molecule that was modified to genetically fuse the two α chains, preventing subunit dissociation, was called rHb1.1, with properties almost identical to DCLHb and $\alpha\alpha$-Hb. This product was abandoned as a blood substitute candidate because of pronounced gastrointestinal side effects and hypertension – effects attributed to NO scavenging. As a consequence, a series of mutants with altered NO affinity was developed (Doherty *et al.*, 1998). A product, rHb 2.2, apparently showed reduced NO affinity but with intact O_2 binding, was free of hypertension in rats, and did not produce microscopic necrotic lesions in monkey hearts (Burhop and Estep, 2001). Some information about this product is available (Burhop *et al.*, 2003), but data have not yet been published to prove that reduced vasoactivity is a result of altered NO binding (Resta *et al.*, 2002).

HEMOGLOBIN SOURCES

Hemoglobin is provided by the red blood cell in highly purified form. However, the red cell contains many enzymes and other proteins, and red cell membranes contain components, such as phospholipids, that could be toxic. Furthermore, plasma proteins and other components could trigger immune reactions in recipients. The chemical modification reactions discussed herein are not specific for hemoglobin, and may modify other proteins as well. Indeed, multifunctional reagents could actually couple hemoglobin to non-hemoglobin proteins.

Rabiner's method (Rabiner *et al.*, 1967) for the filtration purification of hemoglobin was thought to be a significant advance over older centrifugation methods (Brown and Dale, 1936; Hamilton *et al.*, 1947). However, hemoglobin prepared in this way still caused unwanted reactions in human recipients (Savitsky, 1978). The crystallization method (DeVenuto *et al.*, 1977) showed fewer toxic effects in animals (DeVenuto *et al.*, 1977), but batch-to-batch reproducibility was uneven. Ultrapurification of hemoglobin using ion-exchange chromatographic technique is possible, but tedious and expensive (Christensen *et al.*, 1988).

If clinical efficacy and safety of hemoglobin solutions can be shown, the demand for product would soon outstrip the supply of outdated human blood. About 12 million units of blood (1 unit is about 480 ml) are used in the United States each year, and only about 1 per cent outdate. The primary use of blood is in perioperative and emergency settings. The quantity of blood available for use in the production of blood substitutes depends on the willingness of donors who qualify to donate, and the efficient matching of donor blood to recipients.

One solution to the hemoglobin supply problem is to use, as a starting material, blood from non-human sources such as cows (bovine hemoglobin). The ultimate success of bovine (or any

other) hemoglobin depends on demonstration of safety, not supply. One problem in using bovine hemoglobin is the fear of bovine spongiform encephalitis (BSE) agent. This agent, related to the Scrapie organism, has been detected in cows in Europe as well as other mammals in North America. The variant Creutzfield–Jakob Disease (vCJD) has been associated with BSE in Europe, and it is known that BSE can be transmitted by blood in animals. Although there are no known cases of human transmission by blood transfusion at this time, the FDA has placed restrictions on the importation of blood from Europe into the US. Also, at this time there is no adequate test for BSE in donated blood that could be implemented on a large scale.

Under conditions found in the red cell, bovine hemoglobin has a lower oxygen affinity than human hemoglobin because of its greater sensitivity to anions (Bunn, 1971; Feola *et al.*, 1983; Fronticelli *et al.*, 1984, 1986). Thus instead of regulation by 2,3-DPG, as is the case with human hemoglobin, bovine hemoglobin oxygen affinity is regulated by chloride ion.

Bovine hemoglobin has been crosslinked using the bifunctional reagent DBBF to obtain a product with a *P*50 in excess of 40 mmHg under physiologic conditions (Friedman *et al.*, 1984). Although the reaction mixture was somewhat heterogeneous, no uncrosslinked material was detected by sedimentation velocity analysis, and the plasma retention in rats was prolonged tenfold as compared to unmodified hemoglobin. It also has been found that the pyridoxylation reaction raises the *P*50 from 28 to 38 mmHg, and glutaraldehyde polymerization drops the *P*50 to 18 mmHg (DeVenuto, 1988). The polymerized material had essentially the same plasma retention time as human hemoglobin modified in the same way, and rats could also be supported at zero hematocrit.

An alternative and novel source of hemoglobin for modification is from microorganisms, the genome of which has been modified to contain globin genes for recombinant hemoglobin (rHb) production. Significant strides have been made in this approach, and it is possible to express both human α- and β-globin chains in *E. coli* (Hoffman *et al.*, 1990; Looker *et al.*, 1992).

Much of the large-scale development work with recombinant hemoglobin for commercial purposes has been done in commercial laboratories, so not all details of the process are available. However, it is likely that production on the scale needed for a viable blood substitute product could be a problem. One unit of blood (500 ml) contains about 15 g/dl of hemoglobin, so a total of 75 g of hemoglobin would be needed to produce a unit. If the yield is 0.1 g/l of culture medium, 750 l of cell culture would be

EDITOR'S SUMMARY

Hemoglobin, a tetrameric protein, rapidly dissociates into subunits outside of the red blood cell, and these subunits are rapidly cleared in the kidney, causing potentially life-threatening toxicity. Therefore most efforts at chemical stabilization of hemoglobin for use as a blood substitute have focused on crosslinking the subunits to prevent dissociation and prolong the intravascular retention time.

Several broad approaches to modification of hemoglobin have been used, and many variations on each have been studied intensively over the past two to three decades. Available reactive sites include surface lysines and the amino-terminal amino groups and the 2,3-DPG pocket. Specific reagents have been designed to react at these sites and have successfully prevented the rapid renal excretion and subsequent renal toxicity. Recombinant hemoglobins, produced in yeast, bacteria and transgenic animals, have also successfully overcome the problem of subunit dissociation. Several products based on this approach have reached advanced clinical trials, but it is too soon to know whether toxicity (primarily vasoconstriction) has been completely prevented.

A newer approach to hemoglobin modification involves the production of relatively larger molecules that not only prevent subunit dissociation but also interact less with vessel walls to prevent vasoconstriction. These products are still earlier in the pipeline, but show promise.

Whatever product(s) is ultimately successful, supply of hemoglobin as a raw material will become an issue, in proportion to the utility of the product. The most attractive source of raw material has traditionally been human outdated blood, but other potential sources include animal blood or recombinant systems.

needed. In the future it might be possible to express hemoglobin genes in higher organisms; synthesis of functional human hemoglobin has already been reported in yeast, transgenic mice (Behringer *et al*., 1989) and pigs (Swanson *et al*., 1992). However, these approaches present additional purification, logistic and economic problems. Purification of rHb could also be a significant challenge; it would need to be separated from media components and other microorganism products, including endotoxin.

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Chapter 31

Designing Recombinant Hemoglobin for Use as a Blood Substitute

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INTRODUCTION

All blood substitute research is driven by the need for an unlimited and safe supply of an O_2 delivery fluid that would eliminate current problems with supply and storage of donated blood, including typing, disease transmission, limited shelf life, shortages, and unavailability in underdeveloped countries. Most of the potential products are based on solutions of extracellular hemoglobin (Hb) called HBOCs (hemoglobin-based oxygen carriers). These Hb solutions have been designed for resuscitation after traumatic blood loss (hemorrhagic shock), perioperative uses in 'elective' surgery, treatment of septic shock, and transfusion of patients who cannot or will not receive whole blood. The vasoconstrictive activities of current Hb-based products prevent their use in treating strokes and cardiac infarctions. However, if the hypertensive side effect could be eliminated, HBOCs could be used to oxygenate regions of partial arterial blockage in either heart muscle or brain tissue, preventing apoptosis resulting from prolonged ischemia.

GENETICALLY CROSSLINKED HEMOGLOBIN

A large number of reviews and commentaries have been written over the past 7 years discussing the merits, safety, and efficacy of various potential HBOC products (Chang, 2000; 2001; 2003; Gulati *et al.*, 1999; Haney *et al.*, 2000; Alayash, 2001a, 2001b; Stowell *et al.*, 2001; Winslow, 2002; 2003; Yeh and Alayash, 2003; Olson *et al.*, 2004). Unmodified human hemoglobin cannot be used as an extracellular oxygen carrier because it dissociates into dimers, which leads to rapid clearance, oxidative stress and renal damage. The first generation of blood substitutes were designed to prevent tetramer dissociation by crosslinking, polymerizing and decorating hemoglobin with either specific or non-specific chemical agents. These approaches are described in Chapter 30.

In 1992, Looker *et al.* carefully examined the three dimensional structure of human hemoglobin, noted that the C terminus of the α_1 subunit lies 2–6 Å from the N terminus of the α_2 subunit, and then linked the two subunits into a 'di-alpha' unit by inserting a glycine codon into the middle of two fused α genes to span the intervening distance. In order to allow translation in bacteria, the normal N-terminal Valine codon was replaced with AUG for the required N-terminal Methionine in each hemoglobin subunit. The nomenclature for the recombinant hemoglobins is: rHb0.0 ('wild-type') with V1M α and β subunits; rHb0.1, V1M di-α and V1M β; rHb1.1, V1M di-α and V1M β with the Presbyterian mutation (N108K) to raise the *P*50 of the fused tetramer to allow more

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Table 31.1 Distal pocket mutants with reduced NO scavenging and hypertensive side effect

Protein	Distal pocket or allosteric mutations	Helical position or type of mutations	Hypertensive effect (10 per cent topload)	k'_{NOD} ($\mu M^{-1}s^{-1}$)	*P*50 (mmHg)
wt Mb	None			35	0.55
rMb	V68W	E11		4.1	0.91
rMb	L29F	B10		8.1	0.04
rMb	L29W	B10		3.2	21
rMb	L29W/H64Q	B10/E7		2.0	3.4
			%ΔMAP[a] or %ΔTPR[b]		
Native HbA	None		Not determined	75	13
rHb0.1	αβ(V1M) + di-α(1Gly)	Fused di-α	28.4 ± 5.7[a]	58	10
rHb1.1	rHb0.1 + β(N108K)	Allosteric	25.8 ± 2.0[a]	58	32
			33.1 ± 7.1[b]	58	32
rHb1.1Prov	rHb1.1 + β(K82D)	Allosteric	31.7 ± 4.3[b]	78	46
rHbBethesda	rHb0.1 + β(Y145H)	Allosteric	24.1 ± 2.7[a]	56	2.7
rHb3001	rHb0.1 + α(L29F) + β(V67W)	αB10/βE11	7.6 ± 1.0[a]	2	5.2
rHb3011	rHb0.1 + α(L29W/H58Q) + β(V67W)	αE7B10/βE11	2.1 ± 2.2[b]	2	46

The rHb mutants were constructed at BaxterHT based on the myoglobin prototypes listed in the first five rows (see Dou *et al.*, 2002). Ten per cent top load experiments (350 mg/kg) were carried out in a rat blood pressure model (Doherty *et al.*,1998, 2001). The *P*50 values for Mb were measured at 20°C, pH 7, and those for the rHbs at 37°C, pH 7.4. The N108K (Hb Presbyterian) and K82D (Hb Providence) mutations were made to stabilize the *T* quaternary state and raise *P*50 without modifying the active site. Crystal structures of rHb molecules containing αL29W, αV62L, βV67L, β67W, β106W, αL29F/H58Q, and αL29W/H58Q mutations were determined by Eric A. Brucker at Baxter Hemoglobin Therapeutics and Rice University (PDB files 101I through 101P).
MAP = mean arterial blood pressure; TPR = total peripheral resistance.
[a] Data were taken from Doherty *et al.* (1998). Human serum albumin infusion increased MAP 2.5 ± 1.9 per cent in these experiments. The per cent changes in MAP were averaged for 10–90 minutes after infusion.
[b] Data were reported by Doyle *et al.* (2001) as per cent changes in total peripheral resistance (TPR = MAP/cardiac output) measured 50–90 minutes after infusion (Looker *et al.*, 2001). Human serum albumin infusion changed total peripheral resistance (TPR) by −5.5 ± 3.5 per cent.

efficient O_2 transport (Table 31.1; Looker *et al.*, 1992; Chapter 34).

When the di-α and β gene operon is expressed in *Escherichia coli*, only intact tetramers are produced. As in all hemoglobin tetramers, the $\alpha_1\beta_1$ interface is almost covalent in nature when heme is bound and has an estimated equilibrium dissociation constant $\leqslant 10^{-12}$ M (Mrabet *et al.*, 1986a; McDonald *et al.*, 1987; Moulton and McDonald, 1994). As a result, fusion of the α subunits results in the formation of ultra-stable tetramers. The presence of one or two glycines in the linker region does not perturb the tertiary or quaternary structure of hemoglobin and the single glycine linker has little or no effect on the O_2 binding properties of the fused hemoglobin compared to wild-type controls (Figure 31.1; Brucker, 2000). The hemoglobin containing a single glycine linker was transfused into beagles, replacing 30 per cent of the blood volume, and kidney function and histology remained normal, demonstrating that this protein engineering approach to overcoming renal toxicity of hemoglobin succeeded (Looker *et al.*, 1992).

OXYGEN DELIVERY

Removal of human hemoglobin from red cells increases its oxygen affinity due to both loss of the allosteric effector 2,3 diphosphoglycerate (2,3-DPG) and formation of dimers. The importance of oxygen affinity in controlling O_2 transport has been examined theoretically and experimentally in *in vitro* capillary experiments (Boland *et al.*, 1987; Lemon *et al.*, 1987; Nair *et al.*, 1989; Hellums *et al.*, 1996; Page *et al.*, 1998a, 1998b). The rate of uptake is limited primarily by diffusion into the capillary, which is governed by the concentration of dissolved O_2 in the vessel wall and is little affected by the affinity of the O_2 carrier. In contrast, the extent of O_2 release during delivery is directly proportional to the *P*50 of the hemoglobin, which is the primary determinant

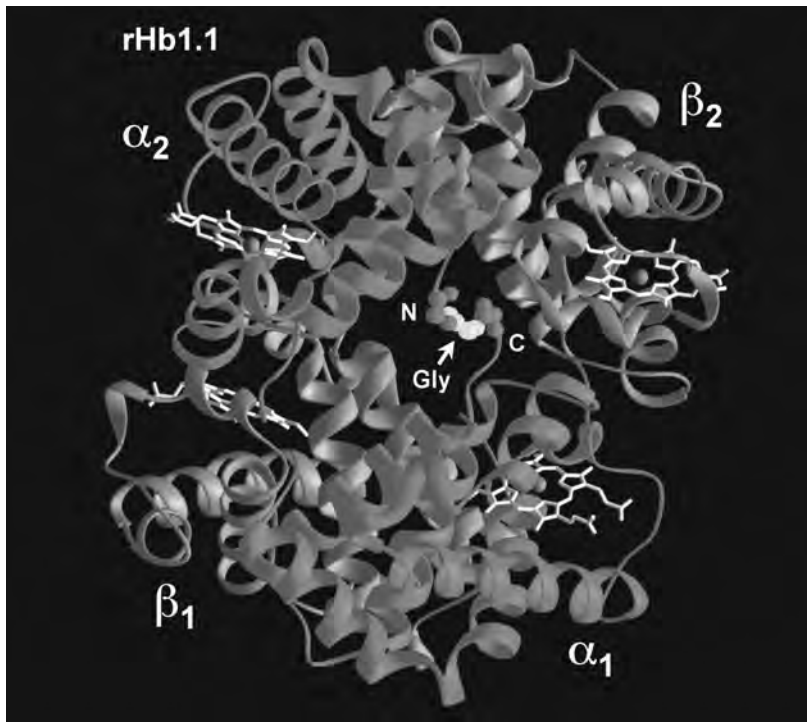


Figure 31.1 Structure of deoxy rHb1.1 showing the single glycine linker between the C-terminus of α_1 and the N terminus of α_2 (PDB accession code 1C7C (Brucker, 2000). See also color plate 7.

of the diffusion gradient from the lumen into the vessel wall. The correlation between *P*50 and the extent of O_2 release holds for native hemoglobin samples, in which *P*50 was varied by chemical modification, pH and organic phosphate addition, and for recombinant myoglobin, in which *P*50 was varied from ~0.1 μM to 30 μM by mutagenesis (Lemon *et* al., 1987; Dou *et al.*, 2002). Hellums, Page and coworkers (Boland *et al.*, 1987; Page *et al.*, 1998a, 1998b, 1999) have also shown that the extent of O_2 release by extracellular hemoglobin is two to three times greater than that by red cells under identical flow conditions due to the uniform distribution of extracellular hemoglobin throughout the lumen of the capillary compared to hemoglobin packaged in red cells, which are concentrated in the center of the capillary where fluid velocity is maximal. These results demonstrate that extracellular Hb-based blood substitutes are inherently more efficient for O_2 transport on a per iron basis and can be used at lower doses than whole blood if they have a comparable *P*50.

Many approaches have been taken to re-adjust the affinity of extracellular HBOCs, based on established mechanisms for ligand binding. A summary of the structural factors regulating O_2 affinity in both hemoglobins and myoglobins is shown in Figure 31.2 and comes from the analysis of several hundred myoglobin and hemoglobin mutants (Olson and Phillips, 1996, 1997; Dou *et al.*, 2002). The reactivity of the heme iron atom can be altered dramatically by stereochemical constraints on the proximal side of the heme group. Large changes in ligand affinity are observed when comparing equilibrium constants for ligand binding to the high affinity (*R*) and low affinity (*T*) quaternary states of human hemoglobin. The *R* to *T* conformational change causes the E and F helices to restrict the ability of the iron atom to move into the plane of the porphyrin ring and react with ligands (Perutz, 1970, 1990). As a result, this quaternary transition causes ~ 1000-fold decreases in the equilibrium constants for the binding of all ligands (Olson and Phillips, 1997; Unzai *et al.*, 1998 and references therein). Examples of using allosteric modifications to increase *P*50 include covalent attachment of pyridoxal phosphate to human hemoglobin (Benesch and Kwong, 1991; Chapter 30), chloride binding to bovine hemoglobin (Fronticelli *et al.*, 1984, 1995; Fronticelli,

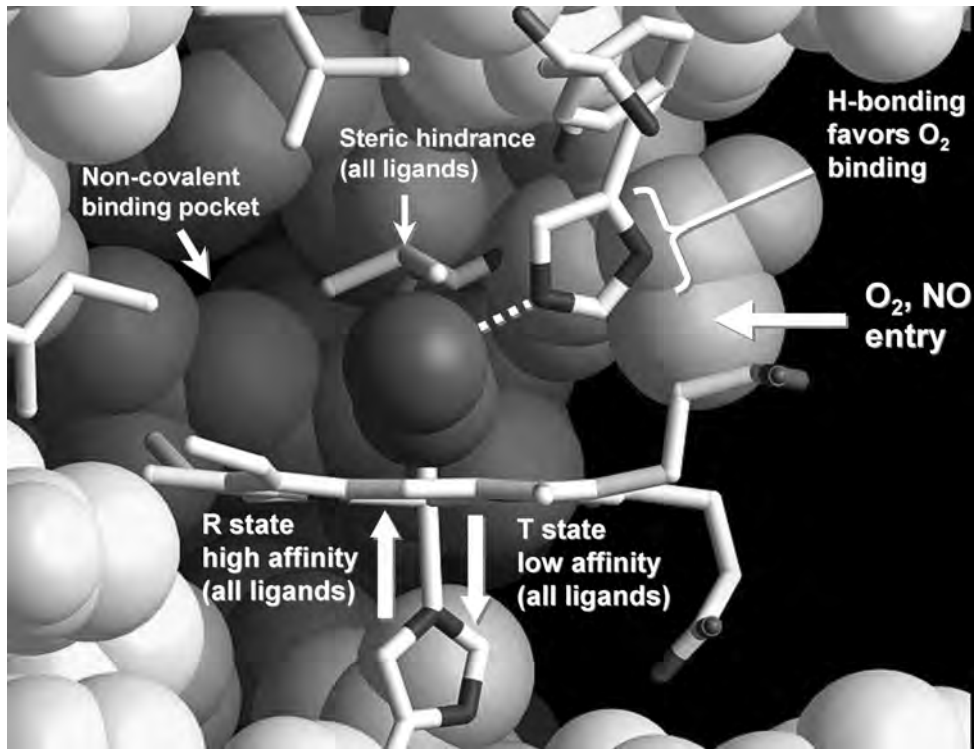


Figure 31.2 The structure of the active site of recombinant sperm whale MbO_2 (Quillin *et al.*, 1993). The amino acid side chains and their helical positons are, starting with the proximal histidine underneath the heme and rotating clockwise: HisF8, Ile(Mb) or Leu(Hb)G8, LeuB10, ValE11, PheCD4, and HisE7, the distal histidine. The non-covalent binding site is where all ligands are captured before bond formation and where they reside before exiting the protein during dissociation. It is also the location for the capture of NO during the dioxygenation reaction. The factors governing O_2 affinity are labeled in white and taken from Olson and Phillips (1996, 1997) and Dou *et al.* (2002). See color plate 8.

1990; Razynska *et al.*, 1990; Chapter 32) and insertion of the allosteric mutations Presbyterian (N108K) and Providence (K82D), which favor the *T* quaternary state (Table 31.1).

Electrostatic interactions with polar side chains on the distal side of the heme group selectively regulate O_2 binding (Springer *et* al., 1994; Olson and Phillips, 1997; Phillips *et al.*, 1999). Amino acids with side chains containing good hydrogen bond donors (i.e., histidine, glutamine, asparagine, tyrosine) favor the binding of O_2 by preferentially stabilizing the highly polar $Fe^{\delta(+)}$–O–$O^{\delta(-)}$, complex but have little effect on the stability of the apolar Fe = C = O complex.The distal Histidine E7 side chains in sperm whale Mb and human Hb form strong hydrogen bonds that selectively stabilize bound O_2 by factors of ~ 300 and ~ 30, respectively. Steric hindrance by large amino acids at the E11 and B10 helical positions can inhibit the binding of all ligands. These strategies can be employed separately or jointly to adjust the *P*50 of recombinant myoglobin and hemoglobin molecules over an ~ 100-fold range (Table 31.1).

THE HYPERTENSIVE SIDE EFFECT

All of the first-generation Hb-based blood substitutes cause some amount of blood pressure elevation (MAP) and gastrointestinal dysmotility (Conklin *et al.*, 1995; Murray *et al.*, 1995; Cullen *et al.*, 1996; Hartman *et al.*, 1998; Gulati *et al.*, 1999; Hess, 1999; Winslow, 1999, 2003; Burhop and Estep, 2001; Konomi *et al.*, 2001; Chang, 2003; Yeh and Alayash, 2003). These hypertensive and gastrointestinal dysmotility side effects clearly reflect interference with smooth muscle relaxation. With the exception of the Winslow and Intaglietta groups (Rohlfs *et al.*, 1998; Winslow, 1998, 2002, 2003; McCarthy *et al.*, 2001), most researchers interpret blood pressure elevation in

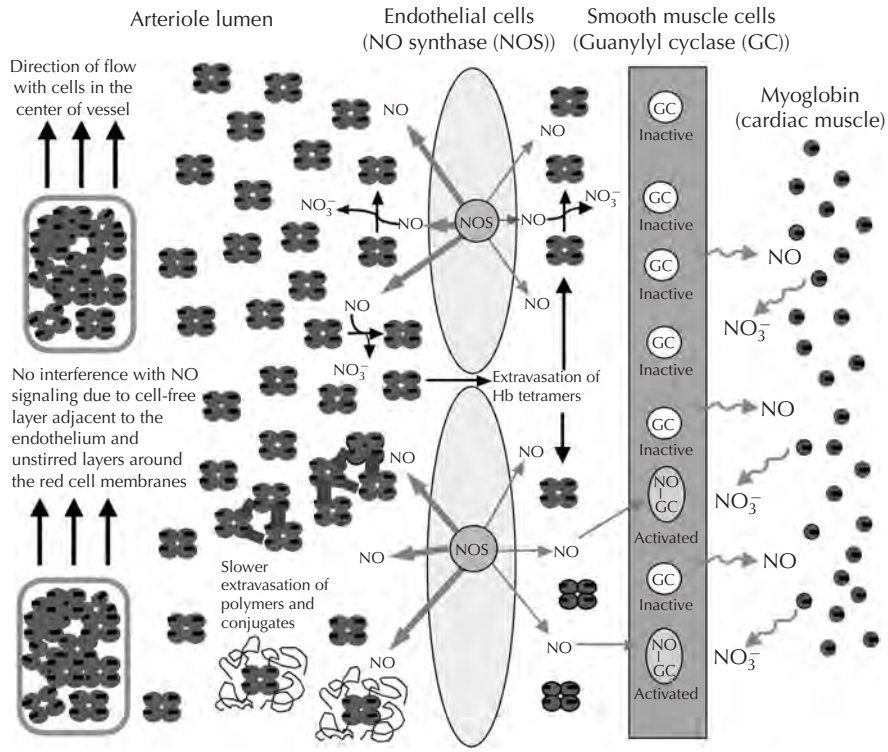


Figure 31.3 Scheme for NO signaling in the endothelium, the roles of intracellular HbO_2 and MbO_2 in detoxifying NO that escapes into the blood stream and muscle tissue, and NO scavenging by extracellular HbO_2 and its effects on smooth muscle function. This scheme is based on the ideas developed over the past 10 years on the physiological role of rapid NO dioxygenation by the oxygenated forms of Hb and Mb (see Brunori *et al.*, 1999; Brunori, 2001; Dou *et al.*, 2002; Olson *et al.*, 2004). NO generated by endothelial cells binds to guanylyl cyclase (GC) in the smooth muscle lining arteriole walls, causing a cascade of events that leads to muscle relaxation. Consumption of NO by extracellular hemoglobin interferes with this signaling, causing sustained constriction. DeoxyHb does bind NO rapidly, but HbO_2 is the major species in arterial blood where O_2 saturation levels are $\geqslant$ 90 per cent. Thus, the major cause of NO depletion is the oxidative reaction of NO with bound dioxygen. The relative importance of luminal NO scavenging *versus* abluminal reactions with extravasated hemoglobin is controversial. At present, most evidence suggests that both reactions affect NO signaling *in vivo*. See color plate 10.

terms of rapid scavenging of NO by extracellular hemoglobin, which prevents smooth muscle relaxation (Blitzer *et al.*, 1996; Doherty *et al.*, 1998; Caron *et al.*, 1999; Erhart *et al.*, 2000; Dou *et al.*, 2002; Chang, 2003). Our view of the mechanism underlying the hypertensive effect and NO metabolism in the endothelium of arterial blood vessels is presented in Figure 31.3.

For oxygen transport by extracellular hemoglobin, the key parameters are total hemoglobin concentration and O_2 affinity (1/*P*50) (Lemon *et al.*, 1987; Page *et al.*, 1998a, 1998b). As long as the association and dissociation rate constants, k'_{O_2} and k_{O_2}, are $\geqslant 0.1\,\mu M^{-1}s^{-1}$ and $10\,s^{-1}$, respectively, the hemoglobin molecules are in equilibrium with free O_2 in their immediate environment because diffusion will be slower than chemical reaction with the iron atom (Lemon *et al.*, 1987; Nair *et al.*, 1990). Large amounts of O_2 are taken up and released in the microcirculation. During release in actively respiring muscle or neuronal tissue, the flux of O_2 out of the capillary lumen is roughly proportional to the *P*50 of the hemoglobin, which determines the free $[O_2]$ diffusion gradient at roughly 50 per cent saturation because the abluminal $[O_2]$ is $\approx$ 0 (Lemon *et al.*, 1987).

Interference with NO signaling involves a competition between the rate of NO oxidation by oxyhemoglobin or binding to deoxyhemoglobin and the rates of NO synthesis in endothelial cells, diffusion into smooth muscle cells, and chemical reaction with guanylyl cyclase. The

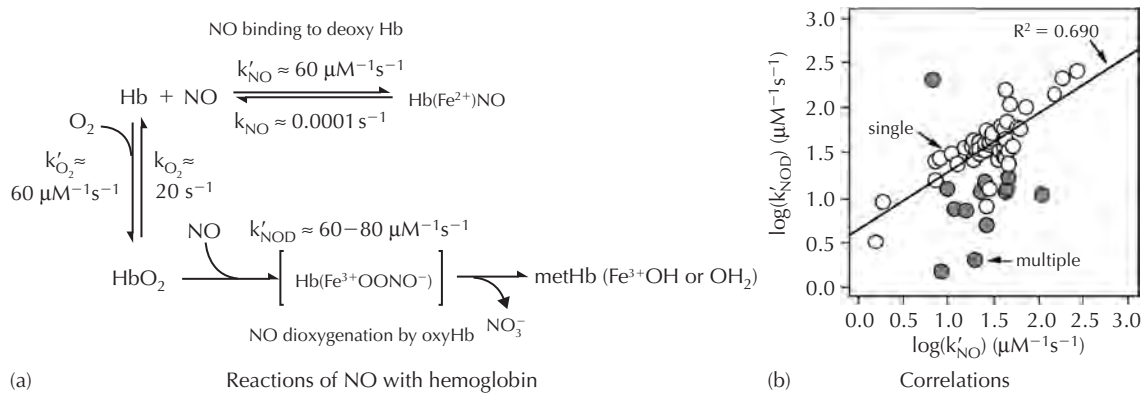


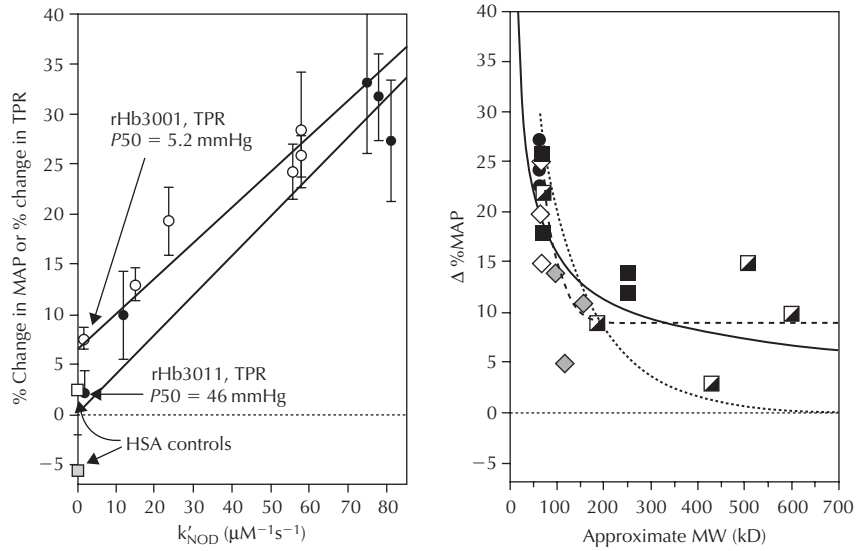
Figure 31.4 (a) Physiologically relevant reactions of NO with hemoglobin (Olson *et al.*, 2004). (b) Correlation between the bimolecular rates of NO binding and the NO dioxygenation reaction for 35 different Mb mutants. Symbols: open circles, single mutants; shaded circles, multiple B10, E7, and E11 mutants of Mb (Eich *et al.*, 1996; Eich, 1997). Note that some of the multiple mutants have much lower k'_{NOD} values than k'_{NO}.

amount of NO present is very small ($\leqslant 1\ \mu M$). The major reaction in arterioles, where the hemoglobin saturation is $\geqslant 90$ per cent, is NO dioxygenation by HbO_2, which is completely irreversible. During this reaction both bound O atoms are incorporated into NO to form nitrate and methemoglobin (see Figure 31.4; Gardner *et al.*, 2001; Herold *et al.*, 2001; Olson *et al.*, 2004). Thus, it is the bimolecular rate constant for NO dioxygenation, $k'_{NOD} \approx 70\ \mu M^{-1}s^{-1}$ (see Table 31.1), and not the affinity of deoxyHb for NO, which determines the extent of interference with NO signaling.

Isovolemic transfusion with whole blood does not normally result in elevated blood pressure because the rate of consumption of NO by intact erythrocytes is limited by diffusion up to and into the cells and is too slow to interfere significantly with vasoregulation during normal flow rates (Liao *et al.*, 1999; Thomas *et al.*, 2001; Liu *et al.*, 2002). However, red cells will remove excess NO that enters the bloodstream due to inflammation or inhalation (Gladwin *et al.*, 2000). Myoglobin detoxifies NO that escapes to the abluminal side of the signaling system (Brunori, 2001a, 2001b). This secondary function for MbO_2 is necessary to prevent inhibition of mitochondrial respiration because submicromolar levels of NO destroy aconitase and inhibit cytochrome oxidase (Brunori *et al.*, 1999; Brunori, 2001a, 2001b; Dou *et al.*, 2002).

Extracellular HbO_2 is much more vasoconstrictive than red cells. In isotropic solutions of cell-free hemoglobin, the intrinsically high rate of reaction of NO with HbO_2 is not limited by diffusion through unstirred layers adjacent to the erythrocyte membrane or through cell-free layers that form at the vessel wall during rapid blood flow. The small hemoglobin molecules can approach the arterial walls and function as a 'sink' for NO. Rapid consumption of NO at the endothelial surface will skew the NO diffusion gradient produced by endothelial NO synthase (eNOS) toward the lumen of the vessel and away from the abluminal guanylyl cyclase receptors located in smooth muscle (Vaughn *et al.*, 1998a, 1998b). More importantly, hemoglobin tetramers can also extravasate into the blood vessel wall and consume nitric oxide in the interstitial space between the endothelium and smooth muscle, directly disrupting the NO signaling process (Kavdia *et al.*, 2002).

The major evidence supporting this interpretation is the strong linear correlation between the observed hypertensive effect of simple genetically crosslinked rHb tetramers and their bimolecular rate constant for NO dioxygenation (k'_{NOD}) measured *in vitro* (Figure 31.5a, Table 31.1; Doherty *et al.*, 1998; Dou *et al.*, 2002; Olson *et al.*, 2004). This correlation holds for molecules with high or low O_2 affinities and when the hypertensive effect is measured as either mean arterial blood pressure or systemic vascular resistance (SVR = mean arterial blood pressure/cardiac output). These results argue convincingly that NO scavenging is the major cause of the hypertensive effect observed immediately after the intravenous administration of extracellular hemoglobin. There is absolutely no correlation between the O_2 affinity and the magnitude of the hypertensive effect for HBOC samples having identical sizes and oncotic properties but with *P*50 values ranging from $\sim$ 2–50 mmHg (Olson *et al.*, 2004). The latter result argues against the



(a) Hypertensive effect vs rate of NO dioxygenation

(b) Hypertensive effect vs Hb size

Figure 31.5 (a) Dependence of the hypertensive effect on the bimolecular rate of NO dioxygenation (k'_{NOD}) measured *in vitro*. Symbols: open circles, %MAP changes for three rHbs with allosteric mutations to vary *P*50 and three rHbs with distal pocket mutations to reduce NO scavenging (Table 31.1; Doherty *et al*., 1998); closed circles, %TPR changes for three rHbs with allosteric mutations to vary *P*50 and two rHbs with multiple distal pocket mutations to reduce NO scavenging and increase *P*50 (Table 31.1 and Doyle *et al*., 2001). (b) Dependence of the hypertensive effect on Hb size (molecular weight, see Olson *et al*., 2004 for more details). Symbols: open diamonds, simple crosslinked Hbs (Rohlfs *et al*., 1998); closed circles, Δ%MAP changes in 10 per cent top-load experiments in rats for rHb tetramers with allosteric mutations (Doherty *et al*., 1998); closed diamonds, Δ%MAP for Hbs decorated with PEG, POE, or *o*-poly-raffinose (Rohlfs *et al*., 1998); closed squares, Δ%MAP for various glutaraldehyde treated rHbs (Doyle *et al*., 1999); half-filled squares, Δ%MAP changes for simple crosslinked Hb (XLHb), polyethylene glycol (PEG)-conjugated Hb, hydroxyethylstarch-conjugated XLHb, polymerized XLHb, and PEG-modified Hb vesicles (Sakai *et al*., 2000). The solid, gray dotted, and black dashed lines represent fits to the following functions, respectively: a power law, a single exponential, and a single exponential with an offset.

alternate model for the hypertensive effect, which suggests that an autoregulatory response overcompensates for increased O_2 transport by extracellular hemoglobin and causes restricted flow in a large number of capillary beds (Rohlfs *et al*., 1998; Winslow, 1998, 2002, 2003; McCarthy *et al*., 2001). Even more importantly, the results in Figure 31.5a (and Table 31.1) show that the rate of NO scavenging (dioxygenation) by hemoglobin can be manipulated independently of the oxygen affinity of the recombinant protein.

The NO scavenging mechanism presented in Figure 31.3 suggests two protein-engineering strategies to reduce interference with NO signaling by an HBOC. The first is to limit extravasation by increasing the size of the hemoglobin molecule (Lew *et al*., 1989; Abassi *et al*., 1997; Vink and Duling, 2000; Matheson *et al*., 2002; Smith *et al*., 2003; Vandegriff *et al*., 2003; Olson *et al*., 2004). There is a strong inverse dependence of the observed hypertensive effect on the size of the HBOC molecule (Figure 31.5b). The large size of the first-generation hemoglobin polymers developed by Northfield Laboratories and Biopure, Inc. almost certainly accounts for their products having lower hypertensive side effects. Similarly, there appears to be little or no hypertensive effect for the second-generation product being developed by Sangart, Inc., MP4, and this lack of interference with NO scavenging is almost certainly due to its large hydrodynamic radius (Manjula *et al*., 2003; Tsai *et al*., 2003; Vandegriff *et al*., 2003, 2004; Drobin *et al*., 2004; Winslow *et al*., 2004). Researchers are still looking for new and novel ways to polymerize native and recombinant hemoglobin to achieve the same reductions in the hypertensive side effect (Fablet *et al*., 2003; Chen *et al*., 2004; Eike and Palmer, 2004).

The second strategy is to reduce the intrinsic rate of NO dioxygenation by re-engineering the active sites of recombinant hemoglobin subunits (Figure 31.5a and Table 31.1). This approach requires a detailed knowledge of the mechanism of NO scavenging by oxyhemoglobin. Only two kinetically relevant reactions occur when NO enters red blood cells or reacts with extracellular hemoglobin: oxidation of NO producing nitrate and metHb, and reversible binding of NO to deoxyHb (Figure 31.4a; Olson *et al.*, 2004 and references therein). The inorganic reaction of NO with free O_2, nitrosation of thiols, and NO binding to residual metHb are all too slow to compete with the direct bimolecular reactions of NO with HbO_2 or deoxyHb, which exhibit rate constants equal to 60–80 $\mu M^{-1} s^{-1}$ (Sharma *et al.*, 1987; Kharitonov *et al.*, 1994, 1995; Eich *et al.*, 1996; Eich, 1997; Olson and Phillips, 1997; Thomas *et al.*, 2001; Dou *et al.*, 2002; Joshi *et al.*, 2002; Olson *et al.*, 2004). Although the fate of ferrous HbNO has been the subject of recent debate (Huang *et al.*, 2001, 2002; McMahon *et al.*, 2002; Gladwin *et al.*, 2003; Luchsinger *et al.*, 2003; Schechter and Gladwin, 2003; Stamler, 2003), we have shown that autoxidation of ferrous HbNO occurs by initial dissociation of NO, rapid rebinding of O_2, and then subsequent reaction of the dissociated NO with bound dioxygen to produce metHb and nitrate (Eich, 1997; Arnold *et al.*, 1999). Thus, all NO that reacts with HbO_2 is oxidized to nitrate, regardless of the fractional saturation of the initial hemoglobin sample. Lancaster, Herold, and their coworkers have argued strongly that any other products are due to the presence of nitrite in the NO solutions or its production by bolus addition of concentrated NO solutions and incomplete mixing (Joshi *et al.*, 2002; Herold and Roeck, 2003). Thus, the key reaction in the hypertensive side effect is NO dioxygenation.

NO DIOXYGENATION BY OXYHEMOGLOBIN

The reaction of NO with either HbO_2 or MbO_2 is very rapid, bimolecular, and, at pH 7, limited solely by the rate of NO entry into the protein, with no evidence for peroxynitrite intermediates (Eich *et al.*, 1996; Herold, 1999; Herold *et al.*, 2001; Olson *et al.*, 2004). Thus, the most straightforward protein engineering approach to inhibit NO scavenging is to reduce the rate of NO capture in the distal pocket of the heme protein.

Placement of large aromatic or aliphatic amino acid side chains at internal positions in the distal pocket of myoglobins and hemoglobins fills the space around the iron atom and inhibits noncovalent capture of all ligands including NO (Scott *et al.*, 2001). These mutations also markedly decrease the bimolecular rate constants for NO dioxygenation (Eich *et al.*, 1996; Eich, 1997; Olson *et al.*, 1997; Dou *et al.*, 2002). Correlations between the rate constants for simple bimolecular NO binding to deoxymyoglobin and those for NO dioxygenation by oxymyoglobin are shown in Figure 31.4, right panel (Eich *et al.*, 1996; Eich, 1997; Dou *et al.*, 2002). Single mutants, designed to fill the distal pocket and prevent ligand entry, seem to have a larger effect on bimolecular binding (as measured by k'_{NO}) than on NO dioxygenation (k'_{NOD}, open circles, Figure 31.4b). However, multiple Mb mutants have been created that selectively reduce NO scavenging relative to O_2 and NO binding (shaded circles in Figure 31.4b). In either case, the reduction in k'_{O_2} is not large enough to limit O_2 transport in the microcirculation. Experimental and theoretical studies have shown that the bimolecular rate of O_2 binding must be reduced to $\leqslant 0.05\,\mu M^{-1} s^{-1}$ before an effect can be seen in capillary O_2 uptake experiments (Lemon *et al.*, 1987; Nair *et al.*, 1989; Dou *et al.* 2002). All of the recombinant hemoglobins with reduced NO scavenging that were tested can maintain normal respiration after complete isovolemic exchange in rat models, at total iron levels roughly half that in whole blood (Doyle *et al.*, 2001; Looker *et al.*, 2001).

Genetically crosslinked human hemoglobin prototypes with reduced NO scavenging were constructed and evaluated by the research group at Baxter Hemoglobin Therapeutics (Table 31.1). The most successful second-generation tetrameric molecule developed by Baxter was rHb3011, which contains Histidine E7 → Glutamine and Leucine B10 → Tryptophan mutations in α subunits, and Valine E11 → Tryptophan in β subunits (Figure 31.6). The two large aromatic substitutions were placed in the distal pocket to decrease ligand capture and inhibit the NO dioxygenation reaction. The optimal location for reduction of NO scavenging is at the B10 helical position in α subunits and at the E11 helical position in β subunits (Eich *et al.*, 1996; Doherty *et al.*, 1998; Dou *et al.*, 2002; Olson *et al.*, 2004). The Histidine E7 to Glutamine substitution in α subunits was added to increase the rate of O_2 dissociation to a value $\geqslant 5\,s^{-1}$, which is sufficient for rapid transport in capillaries (Lemon *et al.*,

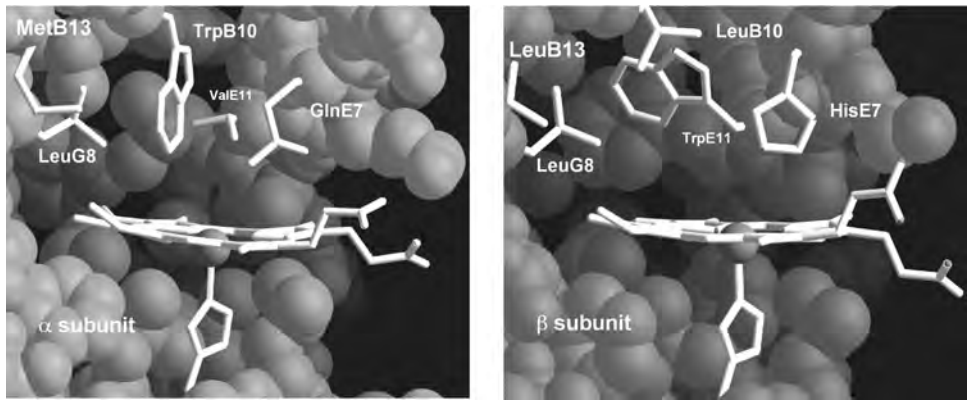


Figure 31.6 Structures of human α and β subunits containing the mutations found in deoxygenated rHb3011. The α heme pocket is from deoxy di-α(V1M, L29W, H58Q)/β(V1M), PDB code 101L (E. Brucker). The β heme pocket from deoxy α(V1M)/β(V1M, V67W), PDB code 101K (E. Brucker). The large indole side chains fill the capture volume in the distal pocket inhibiting NO entry into each oxygenated subunit by ~30-fold. See color plate 11.

1987). In the 10 per cent top load experiments, rHb3011 has virtually no effect on total peripheral resistance compared to the ~ 30 per cent increase observed for the first-generation rHb1.1 molecule (Table 31.1).

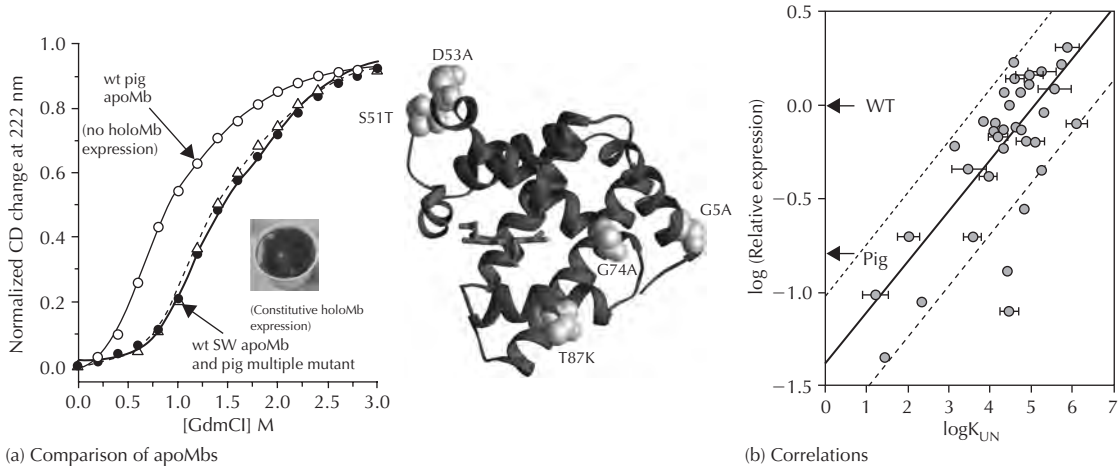
EXPRESSION OF rHB IN *E. COLI*

The major advantage to using recombinant hemoglobin as the starting material for an HBOC is that the intrinsic properties of the molecule can be tailored to fit the requirements of the product and engineered to alleviate or eliminate any harmful side effects. The principal disadvantage is that recombinant proteins are expensive to produce in large quantities and at purity levels required for medical use. In July of 2003, Baxter International suspended its recombinant Hb-based blood substitute project despite the fact that the Baxter Hemoglobin Therapeutics research group had succeeded in engineering recombinant hemoglobins based on rHb3011, which have no significant blood pressure side effects when administered *in vivo* (Figure 31.5; Chapter 34; Doyle *et al.*, 2001; Resta *et al.*, 2002; Malhotra *et al.*, 2003; Olson *et al.*, 2004; von Dobschuetz *et al.*, 2004). The publicly stated reason for Baxter suspending the project was 'failure to meet clinical goals'. Three other considerations were the cost of production, the presence of impurities and degradation products, and the need for further research and development to increase holoprotein expressions yields.

Apoglobin stability and expression

The mechanisms controlling oxygen affinity and NO dioxygenation are well understood and can be used to engineer safer and more effective O_2 transporters. In contrast, the factors governing holohemoglobin expression in *E. coli* are not well understood. Over 10 years ago, we discovered that sperm whale apomyoglobin is 20–100 times more resistant to guanidinium chloride (GdmCl)-induced denaturation than most other mammalian apoMbs (Figure 31.7A; Hargrove *et al.*, 1994; Scott *et al.*, 2000). This observation had been discussed anecdotally in the literature and accounts for why sperm whale apoMb was chosen for detailed unfolding studies (see Hughson *et al.*, 1990, 1991; Barrick and Baldwin, 1993; Eliezer *et al.*, 2000; Garcia *et al.*, 2000; Nishimura *et al.*, 2000, 2003 and references therein). Another key observation is that sperm whale holomyoglobin can be expressed constitutively in *E. coli* without adding exogeneous heme and without producing large amounts of unfolded apoprotein in inclusion bodies (Springer and Sligar, 1987). In contrast, pig, horse and human myoglobin cannot be expressed as holoproteins. Even when heme is added, the yield of these holomyoglobins is poor and most of the protein is found precipitated in inclusion bodies (Varadarajan *et* al., 1985; Springer and Sligar, 1987; Dodson *et al.*, 1988; Lloyd and Mauk, 1994).

The underlying physiological cause of these differences was discovered in a study of the unfolding properties of 13 different mammalian Mbs (Scott *et al.*, 2000). Apomyoglobins from



(a) Comparison of apoMbs (b) Correlations

Figure 31.7 (a) Differences in stability of sperm whale and pig apoMb. CD titration curves are shown in the left panel for unfolding of wild-type pig apoMb (open circles), wild-type sperm whale apoMb (filled circles), and pig apoMb with five replacements based on the sequence of the sperm whale protein: G5A/S51T/D53A/G74A/T87K (open triangles) in the titration curve. The spheres in the ribbons drawing show the location of the mutated residues. The solid and dashed lines represent global fits to the observed CD and fluorescence changes as described in Scott *et al*. (2000). (b) Correlation between the log K_{UN} measured in 200 mM KPi and the log(relative expression level) for 35 single, double and triple mutants of sperm whale myoglobin. This correlation explains 52 per cent of the total variance and has a *P* value of 0.0000009. The linear regression between these two parameters is: log(expression) = −1.26 + 0.27*(log K_{UN}). The dashed lines encompass 90 per cent of the data points and are ± 0.42 from the regression line (Smith, 2003). See color plate 9.

deep-diving whales are significantly more stable than those from terrestrial or surface swimming mammals. These results indicate that there is significant selective pressure for increased resistance of Mb to denaturation during the sustained hypoxic and acidotic conditions that occur in whale skeletal muscles during deep and prolonged dives (Zapol *et al*., 1979; Snyder, 1983; Kooyman and Ponganis, 1998; Tang *et al*., 1998).

Comparisons of the amino acid sequences of pig and sperm whale myoglobin suggested various substitutions that might account for the differences in stability. Five replacements are sufficient to increase the stability of pig apoMb to that of wild-type sperm whale apoMb (Figure 31.7a; Scott *et al*., 2000). The three alanine mutations, G5A, D53A, and G74A, appear to stabilize the native folded state by elongating and strengthening the A, D, and E helices. More importantly these results show that a comparative mutagenesis approach can be used successfully to enhance apoglobin stability and imply that this strategy can increase holoprotein expression in bacteria.

Our working hypothesis is that newly translated apoprotein must remain in solution and be resistant to proteolysis long enough for heme to be made available by either bacterial synthesis or transport of externally added heme. In the simplest model, there is competition between precipitation and proteolysis of the unfolded states and heme binding to the native state. The net rate of holo-heme protein formation should be given by the difference in the rates of these processes:

$$R_{net,holoMb} = k_{heme} \bullet \frac{K_{UN}}{1 + K_{UN}} - k_{degrade} \bullet \frac{1}{1 + K_{UN}} \qquad 31.1$$

where R is the rate of holoprotein formation, K_{UN} is the folding equilibrium constant (unfolded, U, to native, N, transition), k_{heme} is the rate of heme transport or synthesis, and $k_{degrade}$ is the combined rate of aggregation, precipitation and proteolysis. This model is the basis of our current strategies to enhance holoprotein production in *E. coli*. If the fraction of the folded states and the rate of heme transport can be enhanced, holoprotein expression will be increased markedly.

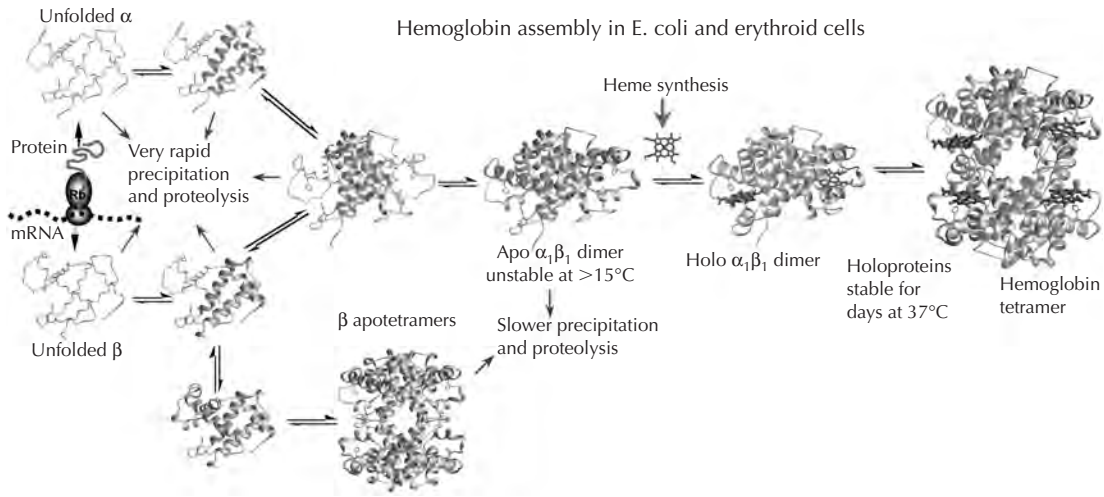


Figure 31.8 Scheme for hemoglobin assembly in both *E. coli* and erythroid cells. The thin lines represent unfolded regions and the large ribbons represent folded helices in the α (silver) and β (gold) subunits. See color plate 12.

Key evidence in support of this approach is shown in Figure 31.7b, where the logarithm of the relative expression of holomyoglobin is correlated with the $\log K_{UN}$ for 35 different myoglobin mutants (Smith, 2003). Statistical analysis suggests that changes in apomyoglobin stability account for 56 per cent of the variance in the expression level. More importantly, these data show that apoglobin stability is necessary but not always sufficient to achieve high production yields. Outliers below the lower 90 per cent regression line represent proteins that have reasonable folding constants but express poorly, perhaps due to higher rates of proteolytic degradation and aggregation, i.e., $k_{degrade}$. In contrast, there are no outliers above the upper dashed line, indicating that no unstable apomyoglobins express well.

Hemoglobin assembly

A scheme for the assembly of holohemoglobin tetramers is shown in Figure 31.8 and based on heme binding and dissociation experiments (Gibson and Antonini, 1960, 1963; Bunn and Jandl, 1968; Antonini and Brunori, 1971; Ascoli *et al.*, 1981; Rose and Olson, 1983; Hargrove *et al.*, 1996, 1997), studies of the hydrodynamic and fluorescence properties of apoHb dimers (Chu and Bucci, 1979a, 1979b; Kowalczyck and Bucci, 1983; Oton *et al.*, 1984; Sassaroli *et al.*, 1984), and measurements of the rates of dimer and tetramer formation and dissociation (Ip and Ackers, 1977; Shaeffer *et al.*, 1984; Mrabet *et al.* 1986a, 1986b; McDonald *et al.*, 1990; Joshi and McDonald, 1994; Moulton and McDonald, 1994). In *E. coli*, the α and β subunits are translated from mRNA that is transcribed from a carefully designed, high copy number plasmid. Apo-α chains do not appear to have any well-formed structure in the absence of a partner β subunit, whereas secondary structure is observed for isolated β apoglobin subunits, which self-assemble into β_4 units at high concentrations, even in the absence of heme (Waks *et al.*, 1973; Oton *et al.*, 1984; O'Malley and McDonald, 1994). By analogy with apoMb, we propose that some nucleation of the G and H helical regions occurs in the apo-subunits to allow formation of a stable $\alpha_1\beta_1$ dimer interface (Figure 31.8). However, the apoHb dimer is much less stable than apoMb monomer and rapidly denatures at temperatures $\geqslant$15°C, even at low concentrations.

The initial bimolecular association rate constant for heme binding to apoglobins is large, $\sim 100\,\mu M^{-1}s^{-1}$, roughly independent of protein structure, and effectively irreversible due to extremely low rate constants for heme dissociation (Gibson and Antonini, 1963; Rose and Olson, 1983; Benesch and Kwong, 1990; Gattoni *et al.*, 1996; Hargrove and Olson, 1996; Hargrove *et al.*, 1996, 1997). The rate constants for holo-monomer to dimer and holo-dimer to tetramer association are between 0.2 and $0.5\,\mu M^{-1}s^{-1}$, and roughly independent of O_2 binding to the heme iron. However, the rate of tetramer to dimer dissociation changes almost a million-fold, from $\sim 1\,s^{-1}$ to $2 \times 10^{-5}\,s^{-1}$ when HbO_2 is

deoxygenated (Ip *et al.*, 1976; Ip and Ackers, 1977) and is the underlying cause of cooperative O_2 binding (Edelstein, 1975; Perutz, 1970, 1990; Ackers, 1980, 1998). In contrast, the rate of holo-$\alpha_1\beta_1$ dimer dissociation, $k_{2,1} \approx 1 \times 10^{-6} s^{-1}$, is little affected by O_2 binding. However, the $\alpha_1\beta_1$ dimer dissociation rate constant does increase markedly, to $\sim 10^{-4} s^{-1}$, in the absence of heme (Moulton and McDonald, 1994). This 100-fold increase in $k_{2,1}$, coupled with the rapid unfolding of separated apo-subunits, accounts for the instability of human apoHb at room temperature (Mrabet *et al.*, 1986a; Moulton and McDonald, 1994).

Using the model for myoglobin expression as framework (Equation 31.1), we have taken three approaches to enhance production of stable holohemoglobin tetramers. These strategies are to enhance the stability of apoHb dimers, co-express the alpha hemoglobin stabilizing protein with hemoglobin subunits to facilitate apo-α chain folding, and increase the rate of heme transport by incorporating a heterologous heme transport system into *E. coli* (i.e., increase K_{UN} and k_{heme} in Equation 31.1).

Comparative mutagenesis of apoHb

One approach to increasing hemoglobin expression is to enhance the intrinsic stability of the apoglobin subunits by mutations based on sequence comparisons between sperm whale and human α and β genes, using the information gained from the same comparison between whale and pig myoglobins (Figure 31.7a). The second approach is to strengthen the $\alpha_1\beta_1$ apoglobin interface based on comparisons between adult β chains and fetal γ chains. The first strategy is based on the assumption that sperm whale hemoglobin is also under selective pressure to be more resistant to denaturation. The second strategy is based on the observation that fetal hemoglobin is significantly more resistant to both acid and alkaline denaturation (Bunn and Forget, 1986). Bunn, McDonald, Adachi, and coworkers have shown that the rate of dissociation of $\alpha_1\gamma_1$ dimers is at least three-fold smaller than that of $\alpha_1\beta_1$ dimers, and that the rate of assembly of holo-α chains with holo-β chains containing G and H helical substitutions based on γ chains can have significantly higher bimolecular rates of dimer formation (Mrabet *et al.*, 1986a; McDonald *et al.*, 1987; Joshi and McDonald, 1994; Adachi *et al.*, 2001, 2003). Thus, it is clear that the replacements found in γ chains significantly stabilize the $\alpha_1\beta_1$ dimer interface, increase resistance to apo-dimer unfolding, and should enhance expression in *E. coli*.

Alpha hemoglobin stabilizing protein (AHSP)

Two years ago, Mitchell Weiss and colleagues identified a 102 amino acid protein whose expression was induced by GATA-1, an essential erythroid transcription factor (Kihm *et al.*, 2002). The expression of this protein progressively increases during normal human erythropoiesis to levels of $\sim$ 0.1 mM in pro-erythroblasts (dos Santos *et al.*, 2004). The protein binds isolated holo-α subunits reversibly with an association equilibrium constant equal to $10\,\mu M^{-1}$ and was named alpha hemoglobin stabilizing protein (AHSP). Holo-β chains can readily displace α subunits from AHSP to form intact hemoglobin (Gell *et al.*, 2002; Kihm *et al.*, 2002; Kong *et al.*, 2004; Santiveri *et al.*, 2004) because the equilibrium constant for the formation of holo-α_1B_1 dimers is $\sim$ 60 000 times greater than that for formation of the AHSP-α chain complex (Figure 31.9). The NMR solution and crystal structures of 'free' and α-subunit bound AHSP have been determined (Santiveri *et al.*, 2004; Feng *et al.*, 2004). The protein forms a remarkably stable three-helix bundle with a surface that appears to mimic the β subunit portion of the $\alpha_1\beta_1$ interface.

Possible functions of AHSP during hemoglobin assembly are shown in Figure 31.9. Weiss and Mackay have speculated that the primary purpose of AHSP is to prevent toxic effects from excess α globin synthesis by binding the α subunits and forming a hemichrome that prevents hemin loss, formation of radical oxygen species, and precipitation (Kihm *et al.*, 2002; Kong *et al.*, 2004). AHSP may also act as a hemoglobin chaperone to facilitate the initial folding of α subunits and allow more efficient and rapid formation of either apo- or holo-$\alpha_1\beta_1$ dimers (Figure 31.9; Kihm *et al.*, 2002; Luzzatto and Notaro, 2002). Newly formed β subunits assemble into apo- or holo-β_4 tetramers (Ip *et al.*, 1976; McGovern *et al.*, 1976; O'Malley and McDonald, 1994) and appear to act as self-chaperones. Much remains unknown regarding AHSP function during erythropoiesis, but the existing *in vivo* and *in vitro* evidence suggests strongly that it facilitates hemoglobin expression. Thus, co-expressing this protein with recombinant hemoglobin has the potential to increase markedly the yield of holohemoglobin in *E. coli*.

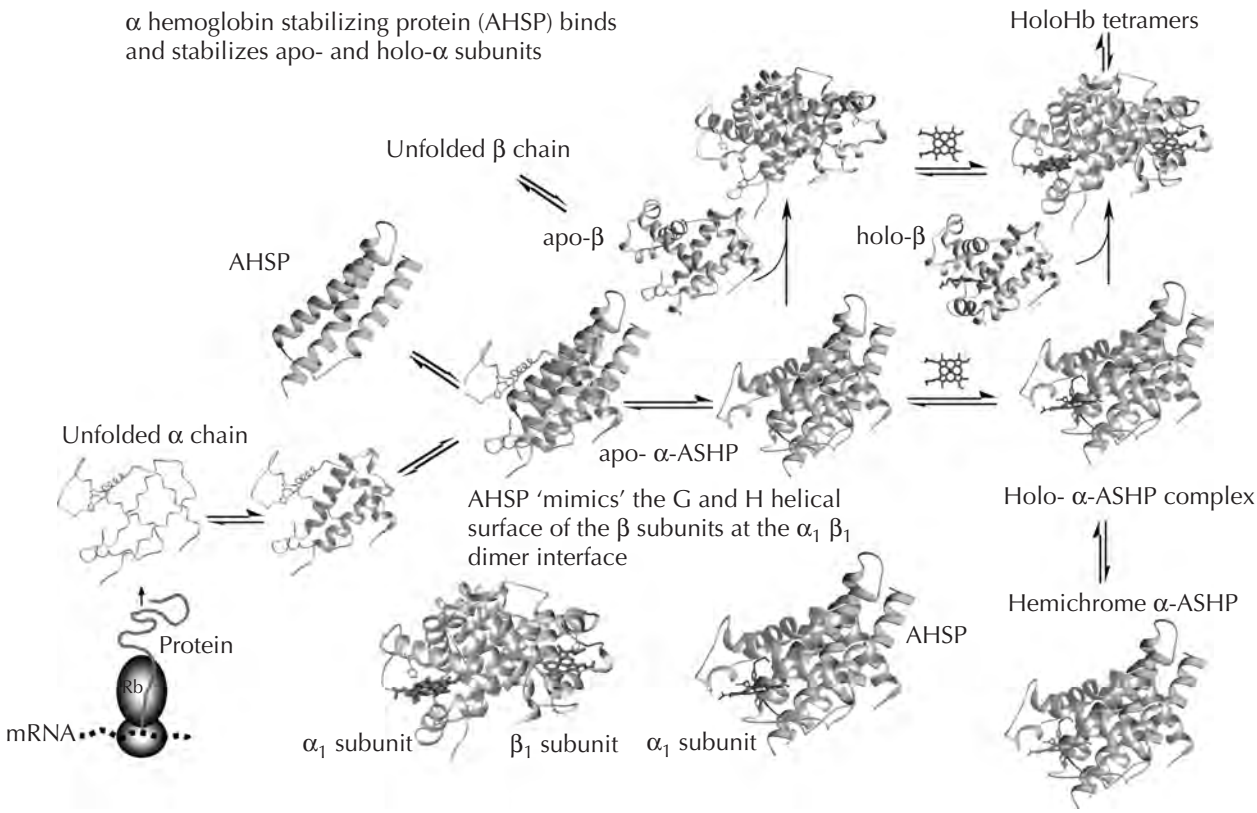


Figure 31.9 Possible functions of α-hemoglobin stabilizing protein (AHSP) in facilitating α chain folding and preventing precipitation, hemin loss, and generation of oxygen free radicals. This scheme is based on the molecular, genetic, functional, and structural work of Weiss, Mackay, Bycroft and coworkers (Gell *et al*., 2002; Feng *et al*., 2004; Kihm *et al*., 2002; Feng *et al*., 2004). The three-helical bundle structure was taken from Feng *et al*. (2004; PDB 1y01). See also color plate 13.

Heterologous heme transport genes

Addition of exogenous heme to bacteria producing myoglobin and hemoglobin is a crude but commonly used way of trying to increase the yield of holoprotein. However, heme uptake by *E. coli* is inefficient, requiring careful selection of the bacterial strain (usually based on JM109 cells) and the addition of a large excess of external heme. Most of the added heme gets 'stuck' in the outer membrane of *E. coli*.

Many pathogenic Gram-negative bacteria have developed efficient transport systems for using heme as an iron source during infections that cause intestinal bleeding. Stojiljkovic and Perkins-Baldwin (2002) have presented an extensive review of the bacterial heme processing genes. Douglas Henderson, at the University of Texas, Permian Basin, has cloned and genetically characterized the heme utilization gene (*hug*) system from the Gram-negative pathogen, *Plesiomonas shigelloides*, by expressing plasmids containing different sets of the *hug* genes and then measuring the extent of heme transport in the transfected *E. coli* (Henderson *et al*., 2001). Diagrams of his system and plasmids are shown in Figure 31.10.

Expression of these heme transport genes is controlled by the *fur* (ferric uptake regulator) transcription factor, which binds iron as a co-repressor and then blocks transcription of the *hug* genes (Bagg and Neilands, 1987; Litwin and Calderwood, 1993; Pohl *et al*., 2003). Iron depletion initiates transcription of the *hug* system. Heme uptake is driven by the TonB energy transduction complex that utilizes a proton gradient to drive active transport of iron siderophores, vitamin B12, and heme across the lipophilic outer cell wall and into the periplasm of most

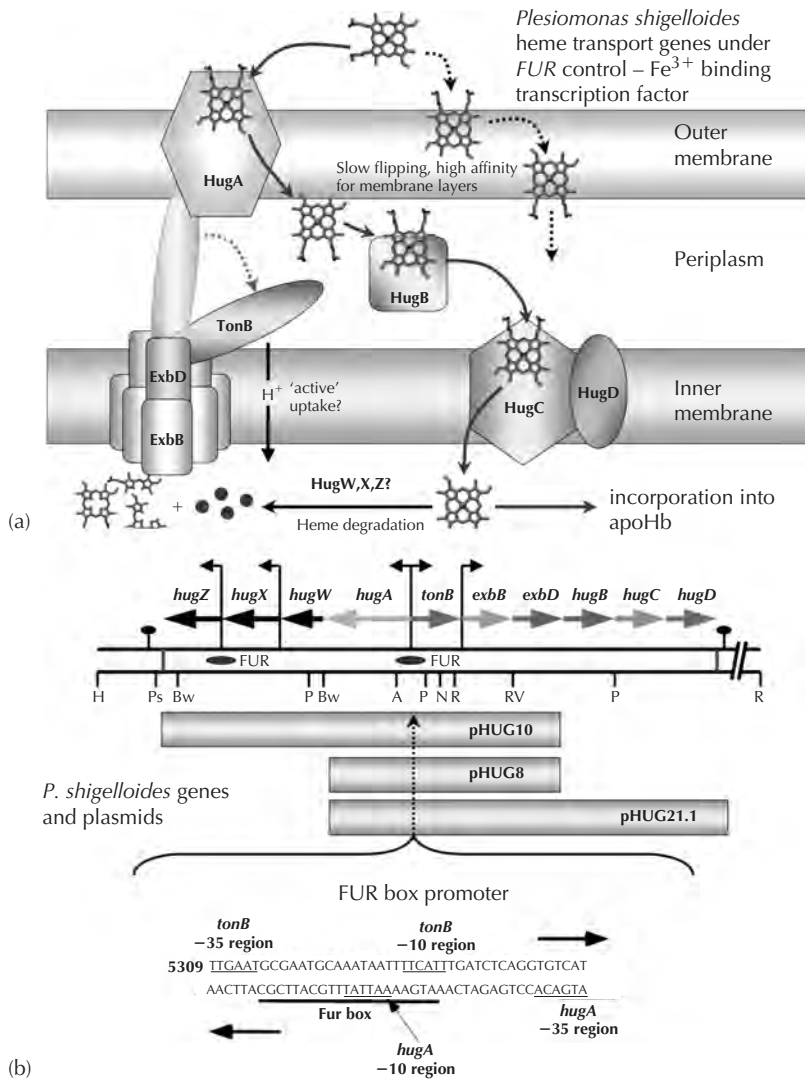


Figure 31.10 (a) Scheme for heme transport in *P. shigelloides* and related pathogens. The structural interpretation of the tonB system was taken from Seliger *et al*. (2001) and Postle and Kadner (2003), and the overall system from Stojiljkovic and Perkins-Balding (2002). Although hemin incorporation into the outer layers of phospholipids membranes is fast, non-facilitated flipping of the heme propionates is very slow. (b) Map of the *P. shigelloides* genes, FUR box and plasmids used for the co-expression experiments with rHb0.0 developed by Henderson (Henderson *et al*., 2001). See color plate 14.

Gram-negative bacteria (Postle and Kadner, 2003; Letoffe *et al*., 2004). Heme is then bound by the soluble HugB binding protein and transported across the cell membrane by HugC and HugD. Little is known about the latter process. The *hugX, Y,* and *Z* genes are involved in heme degradation and iron release and are not required for uptake. These degradation genes are excluded from our co-expression vectors.

In collaboration with Henderson, we are currently using a plasmid that encodes the HugA heme receptor, the TonB/ExbB/ExbD energy transduction system, and the transport genes, HugB, HugC, and HugD transport proteins (pHUG21.1, Figure 31.10b). Our preliminary studies suggest that co-expression of the heme transport genes from a low copy plasmid with rHb genes from a high copy plasmid produce ⩾ two-fold increases in holo-hemoglobin expression when external heme is added. However, much more work is needed to prove that this enhancement will occur in large-scale preparations.

SUMMARY

If extracellular HBOCs become commercially successful as oxygen-delivery pharmaceuticals, then recombinant hemoglobin will eventually be the preferred starting material. The reasons are three-fold. First, well-established biophysical mechanisms and protein-engineering strategies have already been used to solve several key problems in the development of HBOCs. These solutions involved constructing tetramers that are genetically crosslinked to prevent dimer dissociation, have active site or allosteric mutations designed to raise *P*50 and enhance transport, and have smaller distal pockets to inhibit NO dioxygenation and interference with vasoregulation (Figure 31.3, Table 31.1). Second, recombinant hemoglobin represents a source that can be manufactured in unlimited quantities and does not depend on human donations or maintenance of herds of large mammals for blood-letting or slaughter. Third, if polymerization and decoration with polyethylene glycol-like molecules are required for a successful product, recombinant hemoglobin can be engineered to facilitate crosslinking and attachment of synthetic polymers. The dilemma is expense.

In our view, the major reason recombinant hemoglobin is no longer being developed commercially is production cost. Kilogram quantities of highly purified, pyrogen-free hemoglobin must be produced in *E. coli* for $10–20 per gram in order to compete with donated blood or the HBOC products that are still in development. To solve this problem, the expression yield of intact, soluble holo-hemoglobin needs to be increased two- to three-fold over the best current levels. We are addressing this problem by: (1) enhancing the stability of human apohemoglobin by mutations based on sequence comparisons with hemoglobins from deep diving mammals and fetal hemoglobins; (2) assisting the folding of α chains and assembly of hemoglobin by co-expression of the newly-discovered alpha hemoglobin stabilizing protein (AHSP); and (3) increasing the rate of heme incorporation into apoHb by co-expression of efficient heme transport genes. Other strategies to promote expression of hemoglobin include co-expressing hemoglobin with the methionine aminopeptidase (MAP), which can cleave Methionine from the N-termini of α and β chains, allowing synthesis of 'authentic' wild-type hemoglobin with the correct N-Valine residue (Shen *et al.*, 1997; Tsai and Ho, 2002), constructing more stable permuted α and β genes (Fishburn *et al.*, 2002; Sanders *et al.*, 2002), and enhancing bacterial heme synthesis by addition of δ-aminolevulonic acid or altering the biosynthetic genes (Nagai *et al.*, 1997). We are convinced that the production problem can be solved using some or all of these modern protein engineering and molecular genetic approaches.

EDITOR'S SUMMARY

Recombinant DNA technology allows the application of structural and molecular biological tools to solve key problems in the development of blood substitutes. The intrinsic properties of the oxygen carrier can be rationally designed using protein-engineering principles to produce a molecule with virtually any desired characteristic. The structure of human HbA has been used to design a fused di-α gene to prevent tetramer dissociation and renal toxicity. The detailed stereochemical mechanism of oxygen binding has been used to create stable recombinant human hemoglobins with *P*50 values ranging from 1 to 50 mmHg. The rate of NO scavenging by the oxygenated forms of these proteins has been varied independently over a wide range of reactivity by alterations of amino acids in the heme pocket. The latter approach led to hemoglobin molecules with reduced or no hypertension in animal models.

The modified hemoglobin selected by Baxter for its next-generation product was based on these mutants with reduced NO binding rates, but in order to achieve longer intravascular retention it was further modified with PEG. Therefore interpretation of physiological and toxicological effects would be clouded by the necessity to alter multiple properties simultaneously. Unfortunately, Baxter has abandoned its research program and these mutants are not available for independent confirmation of the results.

The supply of recombinant hemoglobin expressed in microorganisms is potentially unlimited using modern bioreactor technology. However, the key unanswered question is how to produce recombinant hemoglobin that is competitive in price with donated units of blood. Improvements are needed to increase expression yields in bacteria for large-scale purification. Genetic engineering approaches can be used to improve the intrinsic stability of globin, to inhibit denaturation before heme insertion, to enhance the rate and extent of subunit folding, and to increase the rate of heme uptake by co-expression of heme transport genes.

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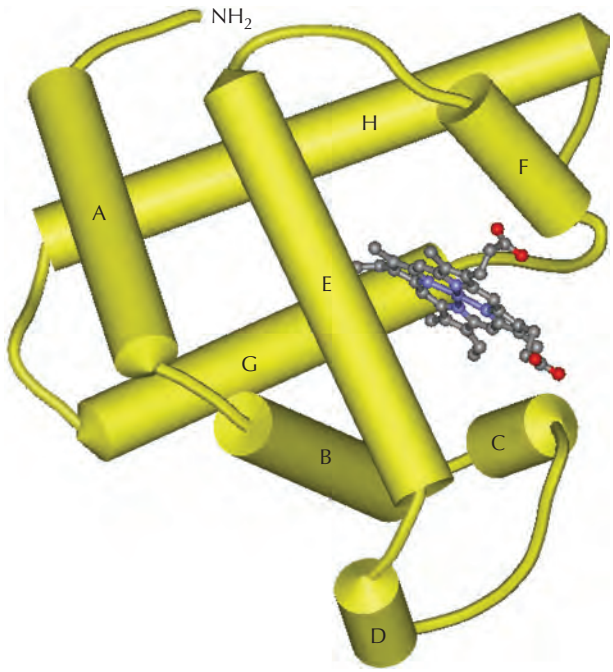


Plate 5 The 'globin fold' as exemplified by sperm whale myoglobin. The eight α-helices are named A through H. See Fig. 29.1.

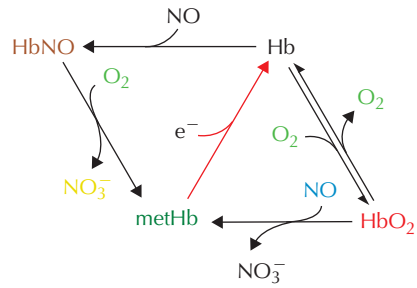


Plate 6 A scheme of the reactions of nitric oxide with the heme iron of Hb and Mb. The formation of a nitroso-thiol with the —SH group of Cys F9(93)β is not taken into account in this scheme. See Fig. 29.5.

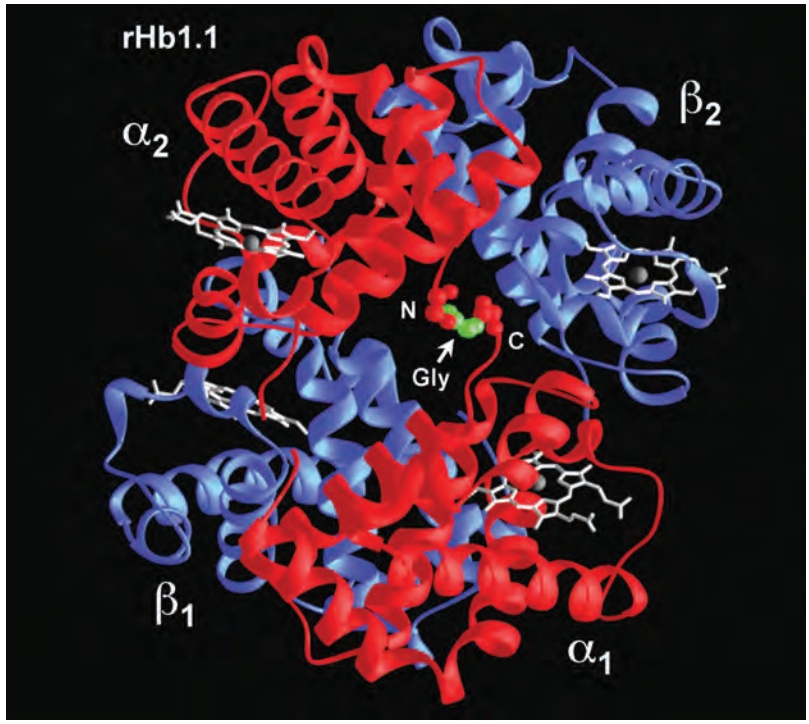


Plate 7 Structure of deoxy rHb1.1 showing the single glycine linker between the C-terminus of α_1 and the N terminus of α_2 (PDB accession code 1C7C (Brucker, 2000). See Fig. 31.1.

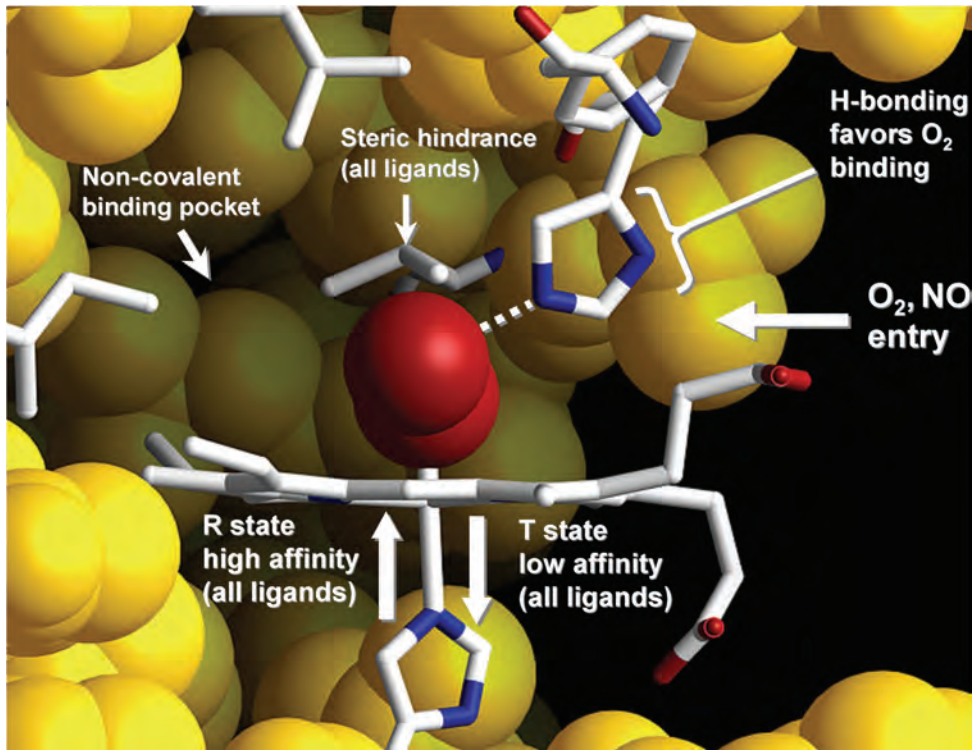
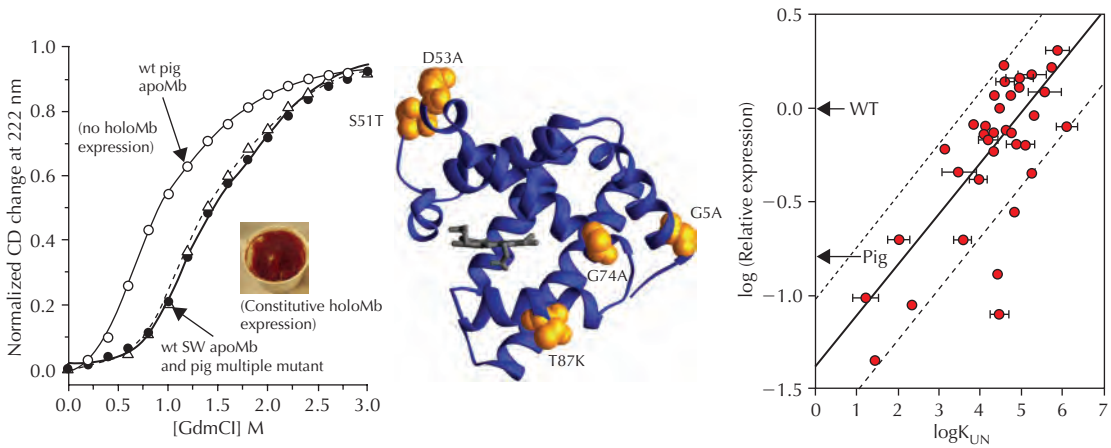


Plate 8 The structure of the active site of recombinant sperm whale MbO_2 (Quillin *et al.*, 1993). The amino acid side chains and their helical positons are, starting with the proximal histidine underneath the heme and rotating clockwise: HisF8, Ile(Mb) or Leu(Hb)G8, LeuB10, ValE11, PheCD4, and HisE7, the distal histidine. The non-covalent binding site is where all ligands are captured before bond formation and where they reside before exiting the protein during dissociation. It is also the location for the capture of NO during the dioxygenation reaction. The factors governing O_2 affinity are labeled in white and taken from Olson and Phillips (1996, 1997) and Dou *et al.* (2002). See Fig. 31.2.



(a) Comparison of apoMbs

(b) Correlations

Plate 9 (a) Differences in stability of sperm whale and pig apoMb. CD titration curves are shown in the left panel for unfolding of wild-type pig apoMb (open circles), wild-type sperm whale apoMb (filled circles), and pig apoMb with five replacements based on the sequence of the sperm whale protein: G5A/S51T/D53A/G74A/T87K (open triangles) in the titration curve. The spheres in the ribbons drawing show the location of the mutated residues. The solid and dashed lines represent global fits to the observed CD and fluorescence changes as described in Scott *et al.* (2000). (b) Correlation between the $-\log K_{UN}$ measured in 200 mM KPi and the log(relative expression level) for 35 single, double and triple mutants of sperm whale myoglobin. This correlation explains 52 per cent of the total variance and has a *P* value of 0.0000009. The linear regression between these two parameters is: log(expression) = $-1.26 + 0.27*(-\log K_{UN})$. The dashed lines encompass 90 per cent of the data points and are ± 0.42 from the regression line (Smith, 2003). See Fig. 31.7.

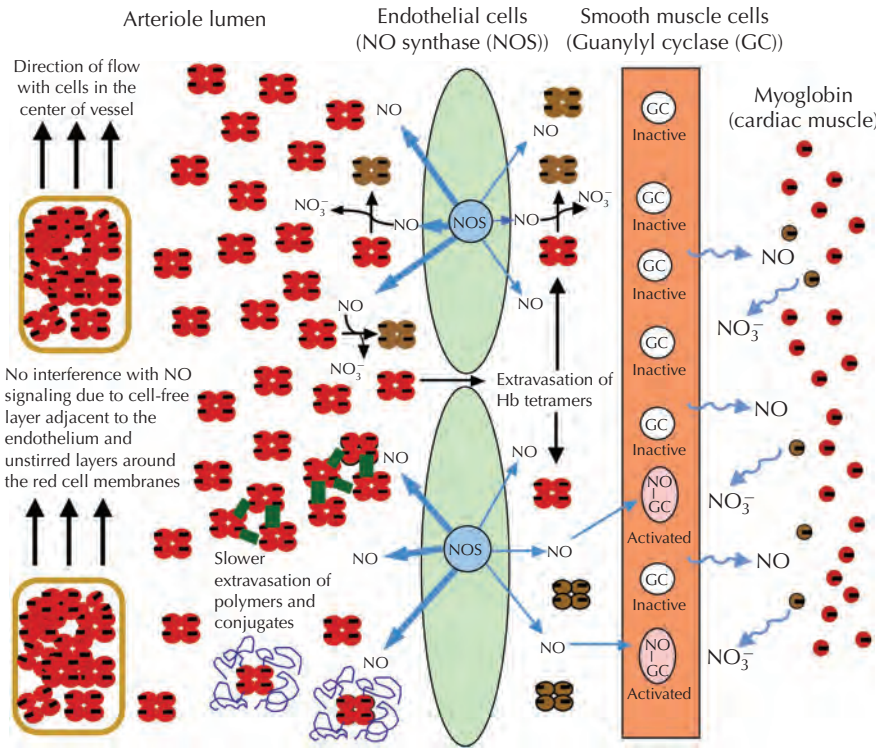


Plate 10 Scheme for NO signaling in the endothelium, the roles of intracellular HbO_2 and MbO_2 in detoxifying NO that escapes into the blood stream and muscle tissue, and NO scavenging by extracellular HbO_2 and its effects on smooth muscle function. This scheme is based on the ideas developed over the past 10 years on the physiological role of rapid NO dioxygenation by the oxygenated forms of Hb and Mb (see Brunori *et al*., 1999; Brunori, 2001; Dou *et al*., 2002; Olson *et al*., 2004). NO generated by endothelial cells binds to guanylyl cyclase (GC) in the smooth muscle lining arteriole walls, causing a cascade of events that leads to muscle relaxation. Consumption of NO by extracellular hemoglobin interferes with this signaling, causing sustained constriction. DeoxyHb does bind NO rapidly, but HbO_2 is the major species in arterial blood where O_2 saturation levels are ⩾90 per cent. Thus, the major cause of NO depletion is the oxidative reaction of NO with bound dioxygen. The relative importance of luminal NO scavenging *versus* abluminal reactions with extravasated hemoglobin is controversial. At present, most evidence suggests that both reactions affect NO signaling *in vivo*. See Fig. 31.3.

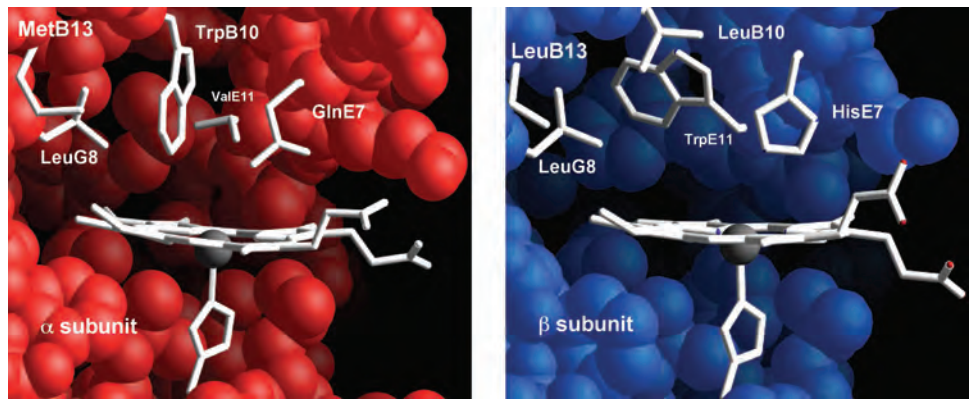


Plate 11 Structures of human α and β subunits containing the mutations found in deoxygenated rHb3011. The α heme pocket is from deoxy di-α(V1M, L29W, H58Q)/β(V1M), PDB code 101L (E. Brucker). The β heme pocket from deoxy α(V1M)/β(V1M, V67W), PDB code 101K (E. Brucker). The large indole side chains fill the capture volume in the distal pocket inhibiting NO entry into each oxygenated subunit by ~30-fold. See Fig. 31.6.

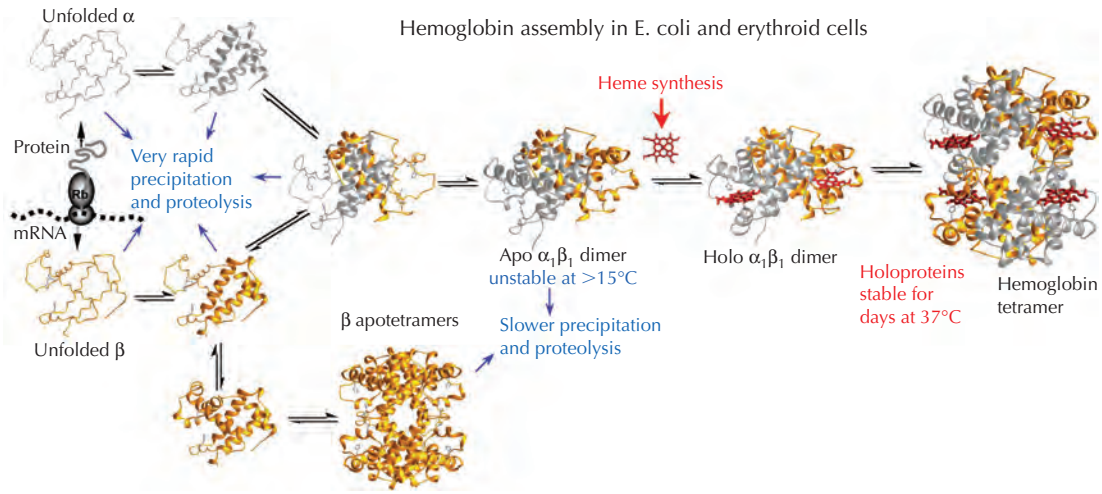


Plate 12 Scheme for hemoglobin assembly in both *E. coli* and erythroid cells. The thin lines represent unfolded regions and the large ribbons represent folded helices in the α (silver) and β (gold) subunits. See Fig. 31.8.

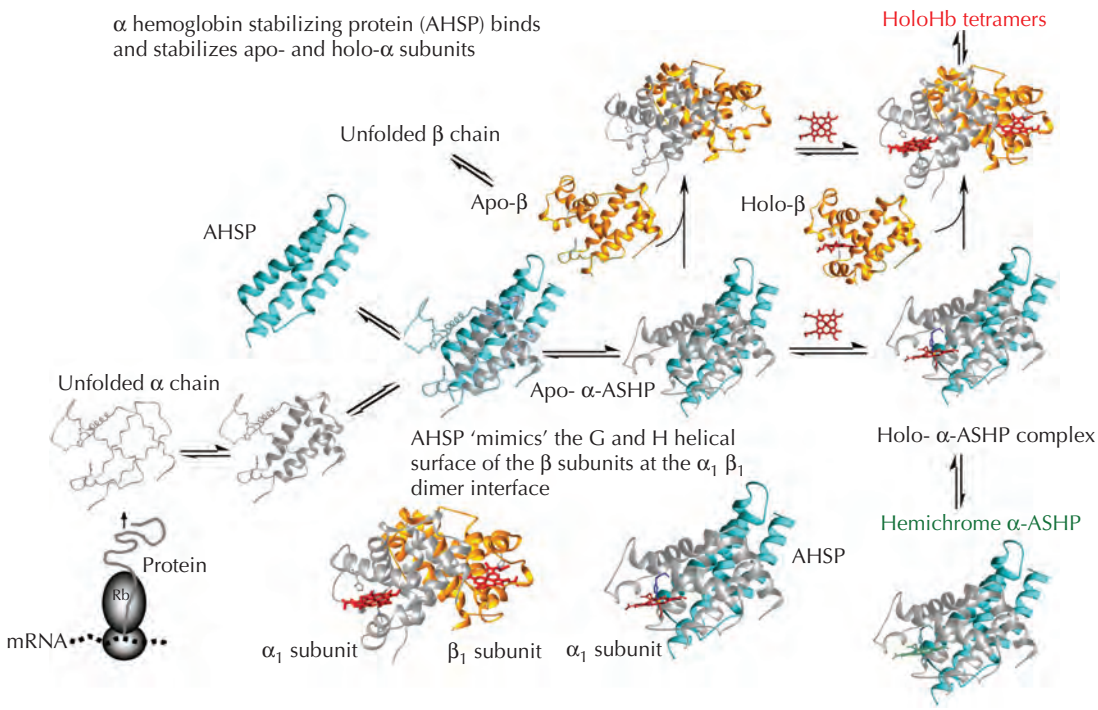


Plate 13 Possible functions of α-hemoglobin stabilizing protein (AHSP) in facilitating α chain folding and preventing precipitation, hemin loss, and generation of oxygen free radicals. This scheme is based on the molecular, genetic, functional, and structural work of Weiss, Mackay, Bycroft and coworkers (Gell *et al.*, 2002; Feng *et al.*, 2004; Kihm *et al.*, 2002; Feng *et al.*, 2004). The three-helical bundle structure was taken from Feng *et al.* (2004; PDB 1y01). See Fig. 31.9.

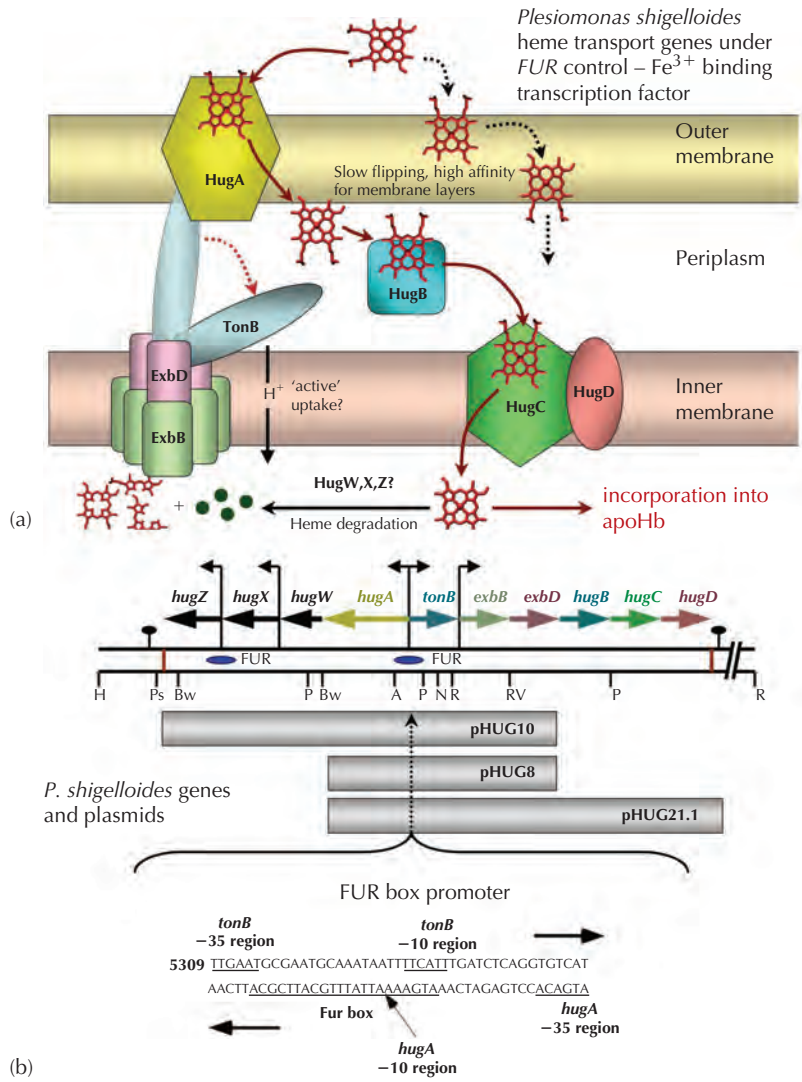


Plate 14 (a) Scheme for heme transport in *P. shigelloides* and related pathogens. The structural interpretation of the tonB system was taken from Seliger *et al*. (2001) and Postle and Kadner (2003), and the overall system from Stojiljkovic and Perkins-Balding (2002). Although hemin incorporation into the outer layers of phospholipids membranes is fast, non-facilitated flipping of the heme propionates is very slow. (b) Map of the *P. shigelloides* genes, FUR box and plasmids used for the co-expression experiments with rHb0.0 developed by Henderson (Henderson *et al*., 2001). See Fig. 31.10.

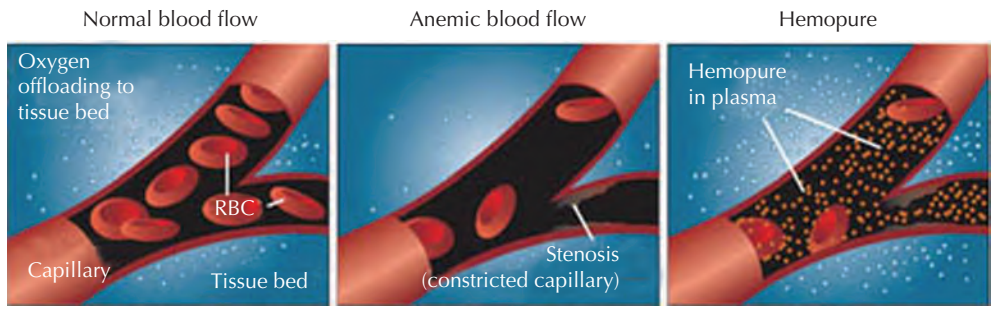


Plate 15 Because HBOC-201 circulates freely in plasma, and is smaller, has lower viscosity (resistance to flow) and can more readily release oxygen to tissues than red blood cells, it can carry oxygen at low blood pressure and through constricted or partially blocked blood vessels to areas of the body that red blood cells cannot reach due to their larger size. See Fig. 36.3.

Chapter 32

Design, Conformational, Functional and Physiological Characterization of Recombinant Polymeric Heme-Proteins

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INTRODUCTION

Genetic engineering offers the opportunity to design and construct various mutant hemoglobins possessing conformational and functional characteristics that render them suitable for use as therapeutic agents in a variety of clinical situations. Although it may not be currently practical to prepare kilogram amounts of hemoglobin (Hb) in this manner, the continuing development of recombinant techniques should eventually enable limitless production of recombinant Hbs suitable for transfusion and free of mammalian infectious vectors. Problems related to the use of Hb solutions for transfusion are the rapid loss of Hb through the kidney's glomeruli (Urbaits *et al.*, 1991; Matheson *et al.*, 2000) and vasoconstriction (Ulatowski *et al.*, 1996; Hess *et al.*, 1993; Hess, 1999). The latter could be caused by nitric oxide (NO) depletion in the wall of the vasculature as a result of hemoglobin extravasation (Kosaka and Seiyama, 1996; Asano *et al.*, 1998). It may also be caused by an autoregulatory mechanism elicited by excess oxygen delivered from plasma-hemoglobin (McCarthy *et al.*, 2001). Another potential problem is that at the physiological colloid-osmotic pressure of human plasma, only a limited amount of Hb may be safely infused; thus, the oxygen-carrying capacity of blood may not be fully restored.

Polymers of tetrameric Hb have the potential advantage of being transfused in much larger amounts, extravasating less across the endothelium and producing less hypertension. In an effort to prevent these effects, polymers of Hb molecules have been produced by chemical modifications using bifunctional reagents to effect intermolecular crosslinking (Gould *et al.*, 1995; Doyle *et al.*, 1998; Carmichael *et al.*, 2000). As with all chemical modifications, these products possess some degree of heterogeneity. However, arterial hypertension was not observed following

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infusion of a chemically polymerized Hb from which lower molecular weight hemoglobins had been removed, and Hb was not detectable in the lymph (Matheson *et al.*, 2002). This result is consistent with the premise that arterial vasoconstriction is caused by extravasation of low molecular weight hemoglobins.

Recombinant hemoglobins as possible hemoglobin-based oxygen carriers (HBOCs) have been produced in several laboratories (Nagai *et al.*, 1985; Fronticelli *et al.*, 1991, 2004; Looker *et al.*, 1992; Shen *et al.*, 1993). A compound, rHb2.0, was developed by Baxter-Somatogen. In the development of this commercial derivative, the rationale was to decrease the NO affinity by introducing mutations in the heme pocket that would decrease the rate of NO binding to hemoglobin (Olson *et al.*, 2004). The α-chains are expressed as a single polypeptide chain, in order to prevent dimer formation, and the final product is reacted with polyethylene glycol (PEG) to increase its molecular volume. This derivative has a *P*50 of $\sim$35 mmHg, and its use is not associated with increased pulmonary vasoreactivity (Resta *et al.*, 2002). Data are not available with regard to heme affinity, autoxidation rate and cooperativity. In the evaluation of this approach, it is difficult to distinguish between the effects of heme pocket mutations and decreased extravasation due to PEGylation.

The rationale that we followed in the design of recombinant HBOC was based on results from our group that indicated a tight correlation between Hb extravasation and arterial hypertension (Ulatowski *et al.*, 1996, 1998; Asano *et al.*, 1998; Matheson *et al.*, 2002). In addition, we wanted to avoid the use of chemical modifications that decreased the yield of protein recovery while increasing the degree of heterogeneity in the preparations. Polymerization of tetrameric Hb was accomplished through the introduction of Cysteine residues at selected positions on the protein surface and subsequent formation of intermolecular S–S bonds. The S–S bonds are about 2.5 Å long, and we reasoned that their accessibility to the reducing agents present in blood would be hindered by the numerous intermolecular interactions resulting from the crosslinks.

We have produced two groups of polymeric heme proteins. The polymers in the first group are homogeneous (molecular weight $\sim$500 kDa) with a *P*50 of 16–18 mmHg and good cooperativity (n = 1.8–2.2) (Fronticelli *et al.*, 2001; Bobofchak *et al.*, 2003). The polymers in the second group are heterogeneous, with major components having MW $>$ 1 million Da, a *P*50 between 1 and 3 mmHg, and no cooperativity (Fronticelli *et al.*, 2004). We have investigated the conformational and functional properties of these high molecular weight recombinant polymers in order to assess their potential as HBOCs. *In vivo* testing indicated that they are stable, do not cause vasoconstriction, and deliver oxygen to hypoxic tissues (Nemoto *et al.*, 2003, Fronticelli *et al.*, 2004).

DESIGN OF RECOMBINANT HEME PROTEIN POLYMERS

Homogeneous polymers

Polymerization of tetrameric hemoglobin molecules was reported in a naturally occurring Hb mutant, Hb Porto Alegre (βSerine9 $\rightarrow$ Cysteine) (Bonaventura and Riggs, 1967), and it occurs through the formation of intermolecular S–S bonds between the external Cysteine residues present at position β9. Using this Hb as a model, polymerization of tetrameric Hb has been obtained by introducing a Cysteine at position β9. We have obtained a polymer of human Hb, Hb Prisca, α_H(C104S) β_H(S9C + C93A + C112G) (Fronticelli *et al.*, 2001), and a similar polymer of a hybrid hemoglobin, Hb Minotaur, α_H(C104S) β_{Bv}(A9C + C93A), containing α-human and β-bovine chains (Bobofchak *et al.*, 2003). This resulting polymer is referred to as Hb Polytaur. Hb Minotaur has a higher expression level than human Hb due to an elevated production of β_{Bv}-chains. To further increase the expression level of the tetramer, a second α-human gene was inserted into the plasmid. In order to prevent the formation of spurious S–S bonds during the refolding and to restrict polymerization to β9 Cysteine, the naturally occurring α104Cysteine, β93Cysteine, and β112Cysteine of HbA were replaced by Serine, Alanine, and Glycine, respectively. In bovine β-chains, an Alanine is present at β112 and substitution at this site was not necessary.

Polymerization is carried out at pH 8.4, with Hb in the oxygenated form. The extent of polymerization was measured by size exclusion chromatography. Figure 32.1 illustrates a typically sized exclusion profile, comparing the elution of Hb Prisca/Hb Polytaur and tetrameric Hb. The two peaks have symmetrical shapes, indicating the presence of major homogeneous fractions with MW of 500 kDa and 64 kDa. Dynamic light

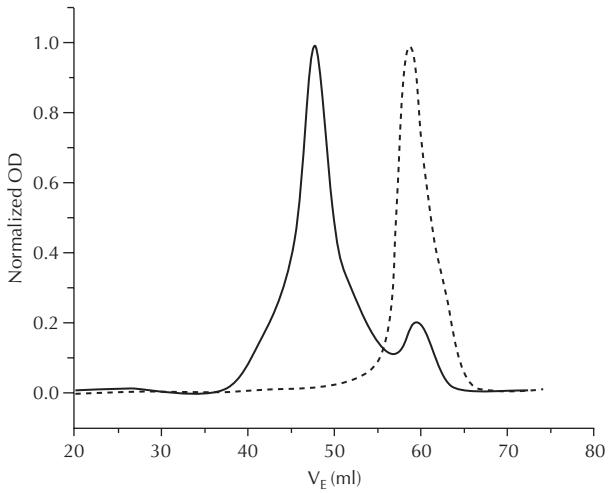

Figure 32.1 Polymerization of Hb Polytaur. Size exclusion chromatograph of Hb Minotaur (- - -) and Hb Polytaur (—) on a prepacked (1.6 × 60 cm) Fractogel EMD BioSec column. The two peaks have symmetrical shapes, indicating the presence of major homogeneous fractions with MW 64 kDa and 500 kDa.

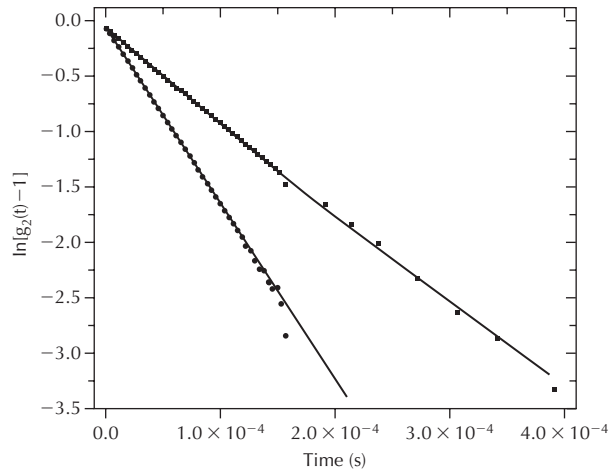

Figure 32.2 Autocorrelation function of HbA (circles) and Hb Prisca (squares) transformed according to the cumulant analysis method. The solid lines are a second-order polynomial fit of the transformed data. The different slopes reflect the different sizes of the two samples. In both cases, the linearity of the slopes indicates the presence of a homogeneous population of molecules (Fronticelli *et al.*, 2001).

scattering was used to investigate the molecular characteristics in solution of Hb Prisca. These measurements were performed, using a DynaPro-801, at a protein concentration of 1–2 mg/ml.

Figure 32.2 shows the autocorrelation function of HbA and Hb Prisca transformed according to the cumulant analysis method. The different slopes reflect the different sizes of the two samples. In both cases, the linearity of the slopes indicates the presence of a homogeneous population of molecules. Figure 32.3 shows the reversibility of the polymerization in the presence of 2 mg/ml sodium dithionite, and the inset shows the excellent correlation between the hydrodynamic radius of the polymer and the number of counts, indicative of the tight packing of the tetrameric hemoglobins, producing a molecule of globular shape. These results are consistent with a polymer comprised of seven to eight non-dissociable tetrameric Hb molecules (Fronticelli *et al.*, 2001).

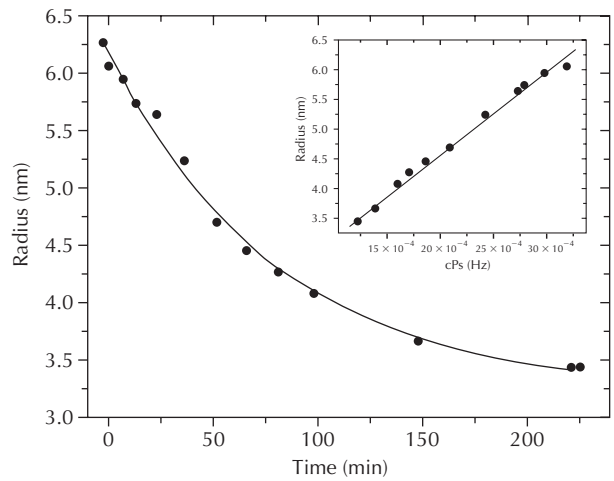

Figure 32.3 Plot of the hydrodynamic radius of the polymer against time following addition of 2 mg/ml sodium dithionite. The inset shows the excellent correlation between the hydrodynamic radius of the polymer and the number of counts, indicative of tight packing of the tetrameric hemoglobins, producing a molecule of globular shape (Fronticelli *et al.*, 2001).

Heterogeneous polymers

When a Cysteine residue is introduced in Hb Minotaur at position β9 ($\alpha_H\beta_{Bv}^{A9C}$), without the substitution of the natural Cysteines present at α112 and β93, a rapid polymerization occurs again by the formation of intermolecular disulfide bonds. This polymer, designated as Hb $(Polytaur)_n$, is heterogeneous, with a large fraction having a molecular weight of 1 million Da or higher. Polymerization is complete after a few hours in the cold (4°C) (Fronticelli *et al.*, 2004). Myoglobin (Mb) polymerization is obtained by introducing

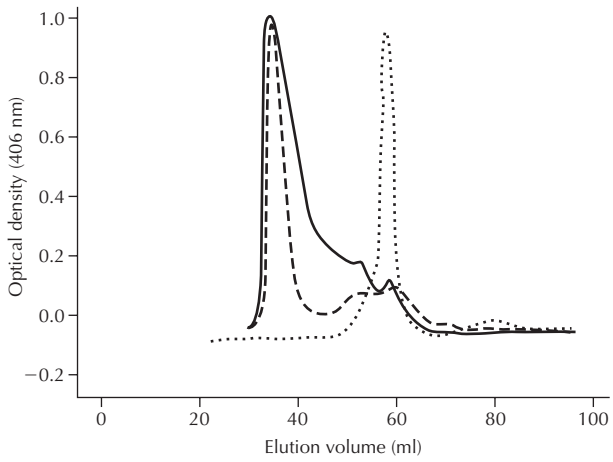

Figure 32.4 Size exclusion chromatography of Hb $(Polytaur)_n$ (—), polymerized myoglobin (▪▪▪) and HbA (- - -) as a control. The elution profile shows the almost complete polymerization of Hb $(Polytaur)_n$ and myoglobin. The polymers are eluted with the void volume of the column, as for derivatives, with a MW ⩾ 1 million Da.

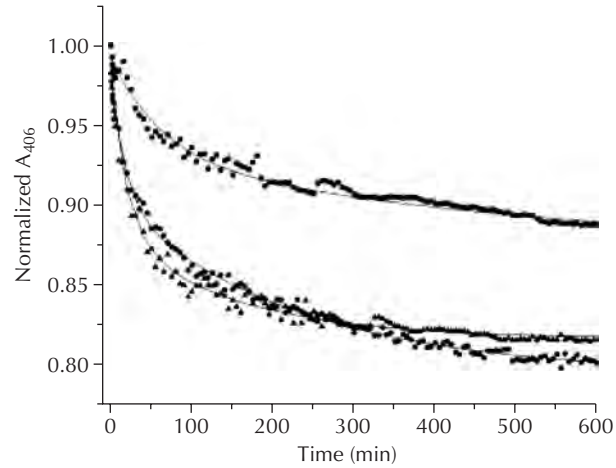

Figure 32.5 Affinity of HbA (●), Hb Minotaur (▲) and Hb Polytaur (■) for heme. The rate of heme loss was the same for HbA and Hb Minotaur. Heme was not released by 40 per cent of Hb Polytaur; in the remaining 60 per cent of the protein, the rate of heme loss was decreased two-fold with respect to HbA (Bobofchak *et al.*, 2003).

into sperm whale Mb the substitutions Glutamine 8 → Cysteine, Lysine 50 → Cysteine, and Lysine 96 → Cysteine. These residues are external, in favorable positions for the formation of disulfide bonds. The replacement of the Lysine residues decreases the Mb molecule net positive charge, favoring intermolecular interaction and S–S bond formation (Fronticelli *et al.*, 2004). Polymerization is rapid and the polymerized product is stable. Gel filtration of Hb $(Polytaur)_n$ and of polymerized Mb is shown in Figure 32.4. In both cases, the major fraction is eluted with the void volume of the column (35 ml), indicating the presence of large polymeric forms with MW ⩾ 1 million Da. Natural HbA was used as control.

BIOCHEMICAL PROPERTIES

Heme affinity and autoxidation rate

Two important aspects in the development of efficient HBOCs are their heme affinity and stability toward oxidation. Rapid heme loss and high oxidation rate diminish the oxygen-carrying capacity of plasma Hb and trigger oxidative stress and cytotoxicity in the endothelial cells (Alayash, 1999; Baldwin, 1999; Baldwin *et al.*, 2003a). Figure 32.5 shows that the rate of heme released by Hb Minotaur was similar to that of HbA. However, heme release was substantially retarded in Hb Polytaur, and 40 per cent of the heme is not released by the protein (Bobofchak *et al.*, 2003). Thus, polymerization, by making the heme less accessible to the solvent, greatly increases the stability of the heme.

With regard to the autoxidation properties, a large difference exists between the rate obtained under *in vitro* conditions and that obtained under *in vivo* conditions. Reducing systems are present in blood, which are expected to affect the autoxidation rate of HBOCs dissolved in the plasma (Snyder *et al.*, 1987). When the measurements were carried out *in vitro*, the half-time of autoxidation of Hb Polytaur was 3.3 hours. However, when the same measurements were repeated with the polymer added to whole blood, the half-time of autoxidation increased to 44 h (Figure 32.6). This represents a 15-fold decrease with respect to the *in vitro* autoxidation rate and implies that, within the ~20-h retention time in the circulation measured in humans with other polymerized hemoglobins (Hughes *et al.*, 1995, 1996), only 25 per cent of the infused Hb Polytaur would be oxidized, while the remaining 75 per cent would stay in the reduced form and remain functionally active as an HBOC.

Stability

Because polymerization is obtained through the formation of intermolecular S–S bonds, it is

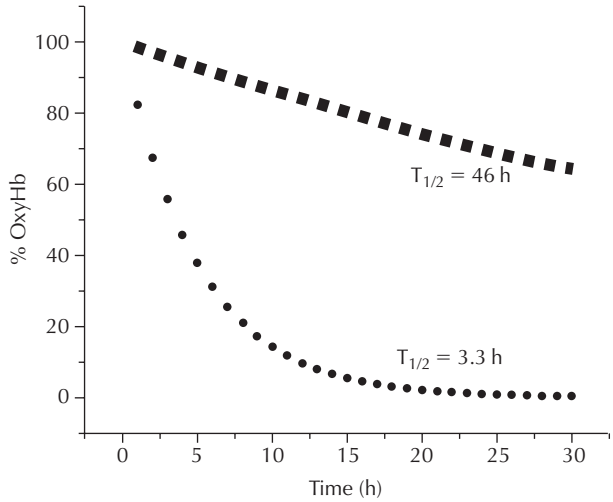

Figure 32.6 Autoxidation rate of Hb Polytaur (circles) in 0.1-M phosphate pH 7.0, T = 37°C and in whole blood (squares). A 15-fold decrease in the rate of autoxidation is observed in Hb Polytaur when in the presence of blood.

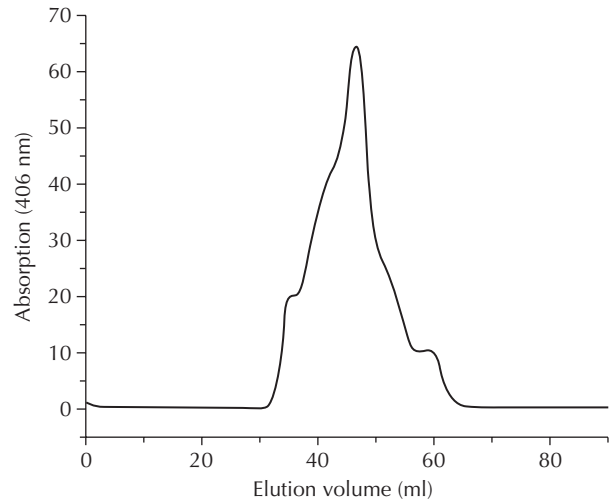

Figure 32.7 Size-exclusion chromatograph of Hb Polytaur after mixing with blood for 24 h at 37°C and corrected for plasma absorbance. The elution profile shows the presence of a main component with MW 500 kDa and the almost complete absence of lower molecular weight polymers, indicating that the intermolecular S–S bonds of these recombinant polymers are not easily reduced when injected in the blood.

important to establish their degree of stability toward the reducing agents present in blood. This property was tested in Hb Polytaur mixed with a ten-fold (v/v) excess of human blood for 24 hours at 37°C. Analysis of a plasma aliquot on a gel filtration column, illustrated in Figure 32.7, shows the presence of a main component with molecular weight 500 kDa and the absence of lower molecular weight polymers. We can conclude that the S–S bonds of these recombinant polymers have a resistance to the reducing agents present in the blood, which is highly compatible with the retention time of polymeric hemoglobins ($T_{1/2} \sim 20$ h) in human circulation (Hughes *et al.*, 1995, 1996).

Oxygen affinity

The optimal oxygen affinity and the relevance of cooperativity for efficient oxygen transport by HBOCs are as yet unresolved (McCarthy *et al.*, 2001). The assumption that HBOCs should have an oxygen affinity similar to that of blood may not necessarily always be the case. One of the advantages of using recombinant hemoglobins and myoglobins as HBOCs is that it is possible to tailor their functional characteristics to different clinical applications by introducing appropriate amino acid substitutions. The recombinant polymers presented here have two ranges of oxygen affinity and cooperativity. Hb Prisca and Hb Polytaur have a *P*50 of ~18 mmHg with good cooperativity, n ~2.0 (Fronticelli *et al.*, 2001; Bobofchak *et al.*, 2003). These values are similar to those of the respective un-polymerized Hbs. In Hb $(Polytaur)_n$ and polymerized Mb, the oxygen affinity is very high, *P*50 of 1–3 mmHg, and cooperativity is absent. Polymerization does not modify the oxygen-binding properties of myoglobin (Fronticelli *et al.*, 2004). As discussed below, we have observed that polymeric Hbs decrease infarction volume in the brain, even when used at low protein concentration (3 per cent), indicating that they function in oxygen delivery. From the results presented in Figure 32.8, it can be projected that Hb $(Polytaur)_n$ and polymerized Mb, at the very low partial pressures of oxygen (1–10 mmHg) existing in hypoxic tissues, have the capability of carrying and releasing oxygen to regions of low O_2 partial pressure.

Nitric oxide affinity

Nitric oxide combines with reduced unliganded Hb without discriminating between the *T* and the *R* allosteric states, and its reaction is virtually diffusion limited, the energy barrier for bond formation being almost nil. Rapid mixing measurements

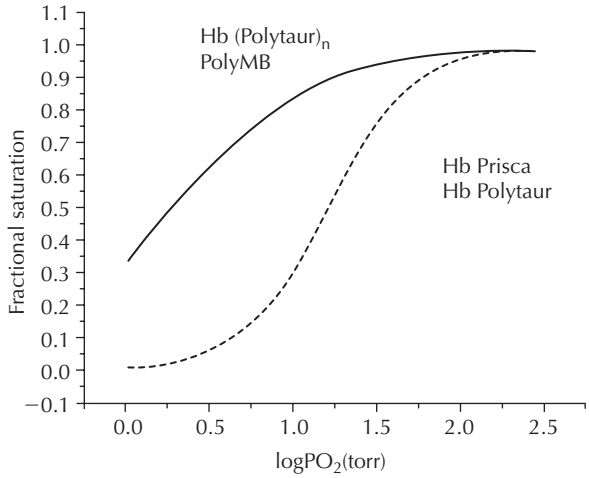

Figure 32.8 Fractional saturation with oxygen binding of Hb Prisca and Hb Polytaur (•) compared to the fractional saturation of Hb (Polytaur)$_n$ and poly Mb. The two groups of polymer show a 10- to 15-fold difference in oxygen affinity.

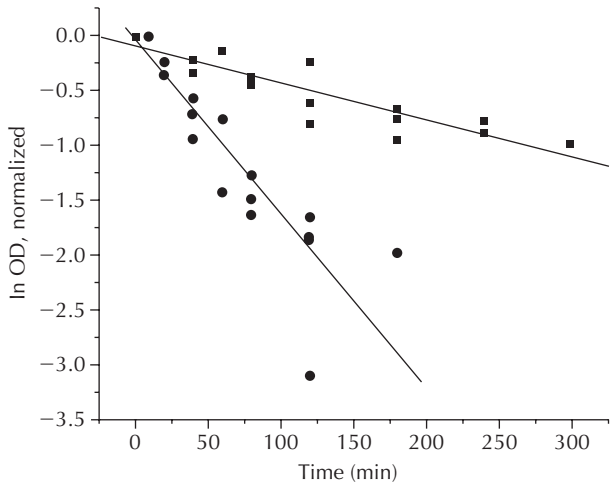

Figure 32.9 Retention time in circulation in mice of HbA and polymeric H/hemoglobins. The infusion volume was 10 per cent of the total blood (~200 µl). The retention half-time was 3.0 h for the polymers (■) and 0.5 h for HbA (●) (Bobofchak *et al.*, 2003).

have been used to probe the rate of diffusion of this gas into the heme pocket. The rate constant for the combination of NO with the deoxygenated forms of HbA and Hb Polytaur is similar, 19–25 $\mu M^{-1}s^{-1}$ (Bobofchak *et al.*, 2003). The fact that Hb Polytaur combines with NO at the same rate as that of HbA indicates that this type of polymerization does not hinder the diffusion of diatomic ligands into the heme pocket.

PHYSIOLOGICAL PROPERTIES

Plasma retention time

Figure 32.9 shows that the plasma retention time of unmodified Hb in mice is 30 min, while retention time of polymerized hemoglobin is increased to 160–200 min. Hemoglobin may be cleared from the plasma in the urinary, lymphatic and reticuloendothelial systems (Bleeker *et al.*, 1986; Keipert *et al.*, 1988). The increase in the plasma retention time as measured in a mouse model was largely due to the lack of clearance in the urinary and, possibly, in the lymphatic systems. Thus, most of the polymer is presumed to be cleared by the reticuloendothelial system and uptake by endothelial cells throughout the circulation. The retention time of Hb polymers varies logarithmically with body mass of the animals. For example, we found that a chemically crosslinked polymer with an average molecular weight of 20 million Da had a half-life of only 200 min in mouse, compared to values of 6 h in rat and 10 h in cat (Matheson *et al.*, 2002). Other Hb polymers that have comparable half-lives in smaller animals have a half-life approaching 20 h in humans (Hughes *et al.*, 1995, 1996). Therefore, we would expect the half-life of our recombinant polymerized hemoglobins in larger animals and humans to be substantially greater than the values observed in the mouse.

Exchange transfusion

Exchange transfusion with a 3 per cent solution (calculated on heme basis) of Hb Polytaur and Hb (Polytaur)$_n$ was performed in mice to produce an approximate 20 per cent decrease in arterial hematocrit. Similar decreases occurred after exchange transfusion of a 5 per cent albumin solution. The plasma concentrations of Hb Polytaur and Hb (Polytaur)$_n$ were 0.5 per cent and 0.2 per cent, respectively, at 24 h after infusion. The exchange transfusion was hypervolumetric (650 µl infused; 350 µl withdrawn) to allow for subsequent blood sampling in the mouse. The hypervolemic exchange transfusion resulted in similar increases in arterial blood pressure in the albumin, Hb Polytaur, and Hb (Polytaur)$_n$ groups over the first 40 min after the transfusion (Figure 32.10). The lack of a substantially greater increase in arterial pressure with Hb Polytaur and Hb (Polytaur)$_n$ than with albumin transfusion implies

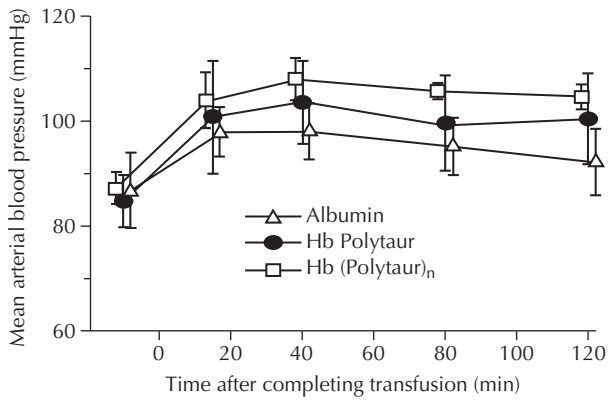

Figure 32.10 Time course of mean arterial blood pressure (±SD) after hypervolemic exchange transfusion with 5% albumin, 3% Hb Polytaur and 3% Hb (Polytaur)$_n$ in anesthetized mice. The increase in pressure was similar among groups over the first 40 min after transfusion, suggesting that the increase was related to blood volume expansion rather than selective NO scavenging in the Hb groups. The increase was sustained in the Hb groups over 2 h, possibly reflecting the longer retention time of the polymers.

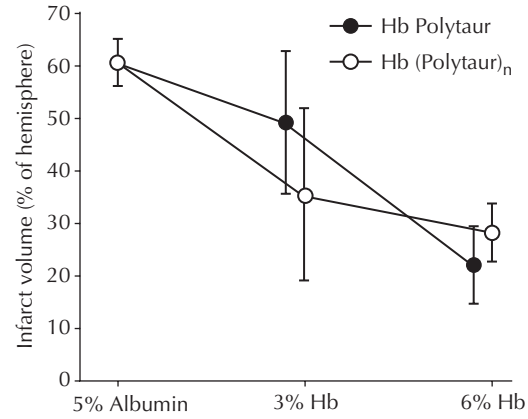

Figure 32.11 Dose–response reduction of cerebral infarct volume (±SD) with exchange transfusion of 3 per cent and 6 per cent solutions of Hb Polytaur and Hb (Polytaur)$_n$ 10–30 min after the onset of 2 h of middle cerebral artery occlusion in mice. Control group was transfused with 5 per cent human serum albumin.

that the increase in arterial pressure is related to volume expansion. Moreover, the polymers do not produce the large pressor response often seen with other hemoglobin solutions. For example, greater increases in arterial pressure were observed in mice transfused with crosslinked tetrameric Hb than with Hb Polytaur (Bobofchak *et al.*, 2002). Polymerization does not hinder NO diffusion into the heme pocket and is unlikely to interfere with binding of NO. Thus, the lack of a large pressor response with the Hb Polytaur and Hb (Polytaur)$_n$ solutions can be attributed to the absence of extravasation of these molecules. The increase in arterial pressure after hypervolemic exchange transfusion was better sustained 2 h after transfusion of Hb (Polytaur)$_n$ than after albumin transfusion (Figure 32.10), possibly reflecting the presence of a residual amount of extravasating forms in Hb (Polytaur)$_n$. Alternatively, it may be due to a more prolonged plasma retention time of this larger polymer. The lack of large decrease in arterial pressure or increase in body temperature implies that endotoxin was effectively removed from these preparations.

Focal cerebral ischemia

We measured the volume of infarction following exchange transfusion with 3% or 6% solutions of Hb Polytaur and Hb (Polytaur)$_n$ during 2 h of transient focal cerebral ischemia produced by middle cerebral artery occlusion with the filament technique in mice (Bobofchak *et al.*, 2003). Dose-dependent reductions in infarct volume were observed with both Hb Polytaur and Hb (Polytaur)$_n$ compared to transfusion of 5% albumin (Figure 32.11; Connolly *et al.*, 1996; Bobofchak *et al.*, 2002; Nemoto *et al.*, 2003). Maximal infarct reduction (64 per cent) was observed following transfusion with 6% Hb Polytaur ($P50 = 18$ mmHg, $n = 2$). When Hb (Polytaur)$_n$ was transfused at either 3 per cent or 6 per cent, infarct reduction was 43 per cent and 54 per cent, respectively. In either case, the data indicate that these polymeric hemoglobins can act as effective oxygen carriers under ischemic conditions.

ENDOTHELIAL PERMEABILITY (EXTRAVASATION)

In the evaluation of HBOCs, it is important to assess the intrinsic effect of these recombinant polymers on endothelial permeability. Studies conducted *in situ* using a rat model following Hb exposure reported an increase in venular permeability with associated neutrophil activation and mast-cell degranulation (Baldwin, 1999; Baldwin *et al.*, 2003b). In our study, we chose to control all aspects of the microenvironment changes associated with the release of secondary

mediators by utilizing endothelial monolayers grown on porous substrates (Dull *et al.*, 2004).

Figure 32.12 demonstrates the ability of the microvascular endothelial monolayer to sieve HbA, Hb Polytaur and Hb $(Polytaur)_n$. The data indicate the presence of an inverse correlation between the size of the hemoglobin species and the concentration of Hb filtered through the endothelium monolayer over time.

The flux of the different hemoglobin derivatives through the monolayer was normalized into a diffusive hemoglobin permeability coefficient (P_{DH}) (Dull *et al.*, 2004). Hb-A increased its own permeability coefficient approximately two-fold during the 4-h exposure period. Hb Polytaur and Hb $(Polytaur)_n$ initially had lower permeability than HbA (Figure 32.13). However, with prolonged exposure, permeability to these polymers also increased several-fold. Hb $(Polytaur)_n$ had the lowest rate of change in P_{DH} during the 1–3-h time interval, but demonstrated a large rate of change from hour 3 to hour 4. The following conclusions can be made: (1) extravasation across the endothelial monolayer is directly altered by

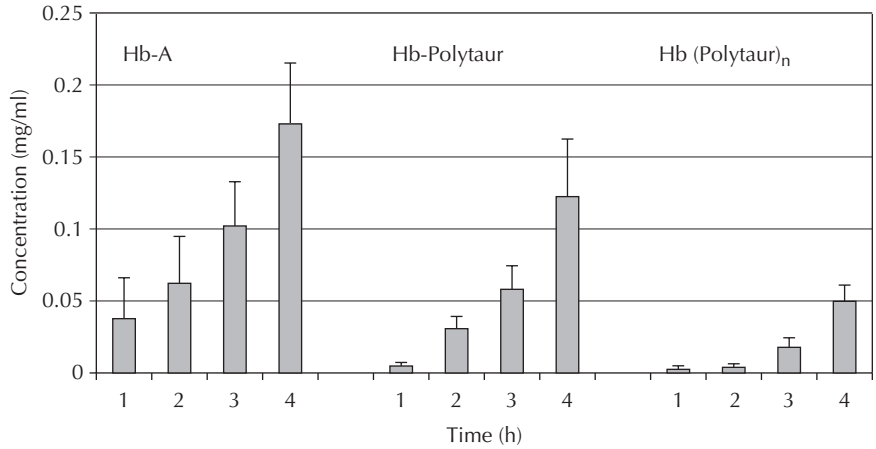

Figure 32.12 Change in hemoglobin concentration (mg/ml) versus time (h). These results show the change in each hemoglobin (Hb) concentration of the hemoglobin filtered through the endothelium monolayer during the 4-h Hb exposure period (Dull *et al.*, 2004).

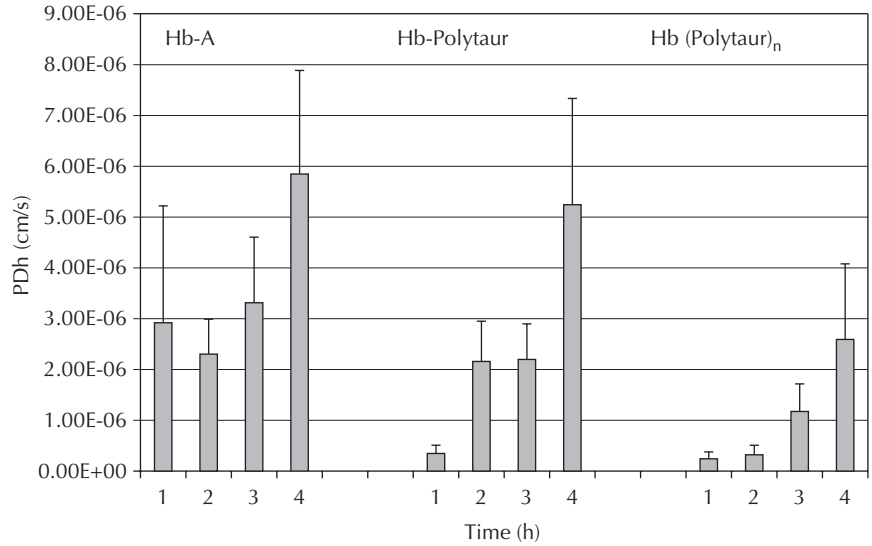

Figure 32.13 Same data from Figure 32.12, normalized into a diffusive hemoglobin permeability coefficient P_{DH}. The three groups have a statistically significant increase in P_{DH} over time ($P < 0.001$); P_{DH} is not statistically different from HbA versus Hb Polytaur ($P = 0.268$), while Hb $(Polytaur)_n$ is statistically different from both HbA and Hb Polytaur ($P = 0.02$) (Dull *et al.*, 2004).

exposure to hemoglobin solutions, and (2) the recombinantly derived Hb polymers eventually induced a greater percentage increase in their own permeability compared to HbA. Thus, it is possible that these polymers induced more endothelial injury as compared to HbA. HbA and Hb Polytaur have similar functional characteristics, and Hb $(Polytaur)_n$ has a higher oxygen affinity. Thus, it is unlikely that the increased endothelial permeability is due to a higher release of oxygen by the polymeric hemoglobins. The greater endothelial injury is probably due to the high autoxidation rate of these polymers. Under the *in vitro* conditions of these measurements, the half-time of autoxidation was 3.3 h for Hb Polytaur versus 33 h for HbA (Bobofchak *et al.*, 2003). On the other hand, the process of autoxidation in simulated *in vivo* conditions is much reduced and the half-time of autoxidation of Hb Polytaur is increased to 46 h (see Figure 32.6). Given that the circulating half-life for polymerized hemoglobins in human is approximately 24 h (Hughes *et al.*, 1995, 1996), autoxidation of these polymers may have a small effect on *in vivo* endothelial permeability. Another important factor associated with oxygen radical production is heme catabolism following heme release (Yeh and Alayash, 2003). Heme stabilization is associated with polymerization (see Figure 13.5); thus, this negative effect should be much decreased in these recombinant polymeric hemoglobins. Size and shape of the macromolecules are important determinants of their diffusion coefficient (D)(Dull *et al.*, 2004). When the P_{DH} are corrected for D, the average $(P_{DH})/D$ values calculated during the 4 hours of the measurements for HbA, Hb Polytaur, and Hb $(Polytaur)_n$ are 5.3×10^{-5} ($\pm 2.5 \times 10^{-5}$), 4.0×10^{-5} ($\pm 2.4 \times 10^{-5}$), 5.5×10^{-5} ($\pm 2.7 \times 10^{-5}$), respectively. These values are statistically similar, indicating that, in the presence of hemoglobin, the endothelium monolayer is altered and loses its sieving power. As a result, the size of the hemoglobin molecules becomes the determinant factor in the regulation of the rate of protein of extravasation.

SUMMARY

We conclude that site-specific introduction of cysteines on the Hb surface permits polymerization, while maintaining physiologically relevant oxygen transport activity, heme affinity, and stability toward oxidation. These polymeric hemoglobins can be used *in vivo* without signs of inducing endotoxic shock or hemoglobinuria. The arterial blood pressure remains stable, consistent with the absence of vasoconstriction. NO diffusion into the Hb Polytaur heme pocket is not hindered by polymerization, and the absence of a large pressor response can be attributed to the absence of extravasation. Reduced ischemic damage in brain suggests that these polymers can act as effective oxygen transporters to ischemic tissues. Possible adverse effects on endothelial permeability have been detected in *in vitro* measurements, probably related to the high autoxidation rate of these derivatives in *in vitro* conditions. These adverse effects are expected to be minimal *in vivo* because autoxidation is decreased in the presence of plasma. Therefore, these recombinant polymers have properties of viable HBOCs that potentially could be developed into a clinically useful transfusion fluid. These data indicate the great applicability of recombinant techniques to the development of HBOC. Following this approach, a variety of stable HBOC products can be obtained in which the size and the functional characteristics are molded to specific clinical applications. The remaining challenge is to obtain highly efficient expression systems in microorganisms. It can be envisioned that this aspect will soon be resolved by the continuous and rapid development of molecular biology.

EDITOR'S SUMMARY

Whatever the precise mechanism of the hemoglobin hemodynamic effect (hypertension, reduced cardiac output and bradycardia), its effect seems to be minimized by elimination of low molecular weight molecules. This is supported by the findings that large molecules, such as polymerized hemoglobin, PEG-modified hemoglobin and dextran-conjugated hemoglobin appear to be much less vasoactive than, for example, $\alpha\alpha$-Hb, DCLHb or rHb1.1. One promising approach to produce a safe hemoglobin-based blood substitute would therefore be to construct molecules that form polymers. This can be done by introducing cysteine residues

strategically located on the surface of the molecule, such that adjacent molecules form disulfide bonds and large polymers. The group led by Dr Fronticelli has developed a human/bovine hemoglobin (Minotaur) which has a molecular weight of 500 kDa, and can be further polymerized if desired.

Studies with Hemoglobin Minotaur of various polymer sizes have been concerned with their effect on permeability of cultured endothelial cells, and have shown that, in general, the larger the size, the less effect. The studies are somewhat clouded by the greater rate of autoxidation of Hb Minotaur compared to hemoglobin A, but this recombinant hemoglobin holds great promise as a model to explore the effects of critical properties such as size, oxygen affinity, diffusion and oxidation rate on toxicity.

ACKNOWLEDGMENTS

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Chapter 33

αα-Crosslinked Hemoglobin

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THE NEED FOR A BLOOD SUBSTITUTE

Establishing a program that would eliminate HIV as a threat to the blood supply became a high priority in the military, and development of a safe and effective 'blood substitute' in 1985 seemed to many an idea whose time had come. Fear was widespread within the US military medical establishment that the HIV epidemic would effectively eliminate the supply of blood for transfusion in wounded soldiers. Since the middle 1960s, US Army medical research teams had worked on a project to chemically stabilize ('crosslink') hemoglobin so as to prolong its intravascular retention and reduce its renal toxicity. In general, the technology that was most successful was to 'polymerize' hemoglobin with glutaraldehyde, a method introduced by Kothe *et al*. (1985) and further refined by the group that would ultimately form Northfield, Inc. Scientists at the Letterman Army Institute of Research (LAIR), San Francisco, had been working on its own version of this product, and had contributed several improvements in the purification procedures under the leadership of Dr Frank Devenuto. The early history of the development of hemoglobin-based blood substitutes has been reviewed (Winslow, 1992a).

As the HIV epidemic expanded, the potential for a blood substitute seemed limitless, and the LAIR group began to plan a transition of their product to larger scale by building a pilot plant capable of producing clinical trial lots of polymerized hemoglobin. In view of the potential for competition with private industry, an alternative plan was devised to contract out a pilot facility. Competitive bids were solicited and after appropriate review, a contract was awarded to Baxter Healthcare in the spring of 1985.

A new group was formed at LAIR in the summer of 1985. Initial assessment of the field by the new team was that the renal toxicity problem had been substantially overcome by completely crosslinking and purifying hemoglobin, but hypertension, first noted critically by Amberson (Amberson *et al*., 1949) remained a mystery. An example was the demonstration, in Dr Valeri's laboratory in Boston, of pressure elevation in the isolated rat heart when it was perfused with hemoglobin solutions (Lieberthal *et al*., 1987). Other problems, which were still not completely solved, included lingering renal toxicity, potentiation of infection, behavioral problems and oxidation (reviewed in Winslow, 1992b). The consensus in the field at the time was that all of these would disappear if the level of purity of hemoglobin could be improved – that is, these effects were believed due to red cell membrane components, not the hemoglobin itself.

The LAIR team felt that the hypertensive effect of hemoglobin must be explained by its structure

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and function. In order to solve the problem, three elements were needed: good physical chemistry, good physiological models and a supply of well-characterized hemoglobin. Recruiting new members of the Division easily solved the first two.

As work under the Baxter contract commenced, two potential products were considered. The first was the traditional glutaraldehyde-polymerized hemoglobin, which had been the focus of studies at LAIR and at other laboratories. This product, however, was inherently heterogeneous, and interpretation of physiological studies would be difficult or impossible. The second was hemoglobin crosslinked between the α chains with bis(dibromosalicyl)fumarate (DBBF). The latter was selected for several reasons, including better homogeneity of the product and oxygen affinity similar to that of blood. It also appeared that interpretation of toxicological and physiological experiments would be easier than with a more heterogeneous product.

The Army called this product DBBF-Hb and, later, αα-Hb. It is the same product that Baxter would call Diaspirin crosslinked hemoglobin (DCLHb) and HemAssist™. The crosslinking chemistry had been discovered by a graduate student (Joseph Walder) working in the laboratory of Dr Irving Klotz (Walder *et al.*, 1979). Walder had refined the chemistry and characterized the product, proposing it for use as an antisickling agent (Walder *et al.*, 1980). Both the Baxter and LAIR laboratories also had experience with the chemistry, and had shown that if the reaction was carried out in completely deoxygenated conditions, the oxygen affinity of the resulting derivative was very close to that of native red blood cells (Walder *et al.*, 1982). Furthermore, the product appeared to be quite homogeneous and easy to characterize (Chatterjee *et al.*, 1986). This seemed the obvious choice for a model compound, and the decision was made for Baxter to scale up its production under the Army contract.

During the 4 years of the Baxter relationship, LAIR received no product for its vasoactivity studies. Baxter, apparently convinced of the ultimate potential of αα-Hb, focused primarily on production scale-up, while LAIR was focused entirely on resolution of vasoconstriction and other biological problems.

Left without product to study, the LAIR team convinced the Army Medical Research and Development Command to build a small pilot plant so that research could continue into the mechanisms of vasoconstriction. A key element of LAIR's proposal to the Army was that it would provide, free of charge, product to scientists who already had research funding in place. Implicit in this commitment from the Army was the agreement that the LAIR laboratory would not compete with any of the companies attempting to commercialize their blood substitute products. On the eve of Operation Desert Storm, LAIR devised a pig protocol to simulate battlefield injury (Hess *et al.*, 1991), and concluded that both unmodified hemoglobin and αα-Hb were excessively toxic (vasoactive) to be considered as blood substitute candidates (Pool, 1990).

To summarize the state of the art at the end of the 1980s, a review (Winslow, 1989) on the subject commented on each of the major unanswered questions at that time:

1. What should the optimal Hb concentration be?
2. What should the oxygen affinity (*P*50) be?
3. What is the importance of the Bohr or CO_2 effects?
4. What should the viscosity of the hemoglobin solution be?
5. What should the desired colloid osmotic (oncotic) pressure be?

This marked the end of the full-scale effort by the Army to produce a blood substitute. The laboratory remained active for several years, supplying hemoglobin solutions to academic researchers and finishing its studies on αα-Hb. In the last 2 years of its activities, the LAIR laboratory produced a number of studies using its αα-Hb, most with negative results (Hess, 1995).

In the end, Baxter and LAIR produced two versions of the same molecule. In general, the degree of vasoconstriction found and reported by the LAIR group led to its elimination from serious contention as a blood substitute (Hess and Riess, 1996). The Baxter version, however, eventually was tested in human clinical trials, but in the end they too reached the conclusion that the product was too vasoactive to be successful (US Public Health Service, 1999). A key point in Baxter's application to the FDA for Phase III trials was that its product and the Army's were fundamentally different. No side-by-side comparison of these two products, however, was publicly disclosed. This chapter will describe the US Army's program at LAIR in development of αα-Hb. Other chapters in this book (23, 34, 35) deal with various aspects of the Baxter program.

PRODUCTION

Purity of hemoglobin solutions has been a significant problem, as suggested by the early work of Rabiner (1975), who showed that rigorous removal of red cell membrane materials reduced toxic effects. In the period 1985 to 1991, intense efforts were expended in commercial as well as military laboratories to purify hemoglobin in order that meaningful toxicological studies could be performed. As an example of the importance of this approach, Burhop *et al*. (1992) were able to show that infusion of purified DCLHb into sheep did not produce the inflammatory responses that were characteristic of solutions contaminated by membrane material.

The production of αα-Hb has been well documented, both by the US Army (Winslow *et al*., 1991; Winslow and Chapman, 1994a; Highsmith *et al*., 1997) and Baxter (Nelson *et al*., 1992a, 1992b; Nelson, 1998). Although the same chemical derivative can be produced using bovine (Fronticelli *et al*., 1986) or other hemoglobins, the commercial product was made using outdated human blood as raw material (see Figure 33.1). In the Army process, blood was not used if it was outdated more than 1 day.

Red blood cells were repeatedly washed with sterile saline to remove all traces of plasma, and then ruptured by gentle hypotonic lysis. The membrane material remaining in the hemolysate was removed by filtration. The starting material for the crosslinking reaction was further purified

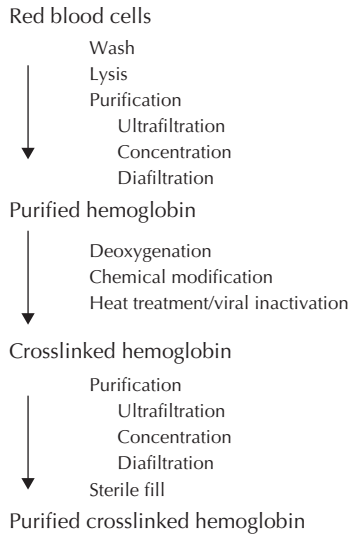


Figure 33.1 Flow chart for the production of human hemoglobin crosslinked between the α chains αα-Hb. From Hess *et al*., 1993.

by various chromatographic techniques, but in general this reduced overall yield with no improvement in vasoactivity (unpublished data). The Army claimed an overall yield of 58 per cent (Highsmith *et al*., 1997), while the Baxter process had an overall yield of approximately 55 per cent (Azari *et al*., 1994).

In order to obtain hemoglobin molecules specifically crosslinked between the Lysine 99α residues, the hemoglobin had to be completely deoxygenated at the time of the reaction (Walder *et al*., 1982). This was perhaps the most difficult and critical step in the manufacturing process, since the affinity of hemoglobin for oxygen is so high. Development of effective gas exchangers for this step occupied a significant amount of time and effort, but the step is critical, since the highest rate of oxidation of hemoglobin occurs at partial oxygen saturation levels (Zhang *et al*., 1990). In order to reduce the oxygen affinity and accelerate the rate of deoxygenation (and to block the 2,3-DPG pocket), an allosteric effector, sodium triphosphate (STP), was added during the deoxygenation step. Finally, the reagent bis-(dibromosalicyl)-fumarate (DBBF) was added, and the reaction was allowed to proceed.

The crosslinked hemoglobin (αα-Hb) was more stable than uncrosslinked hemoglobin (Yang and Olsen, 1991), and this property was exploited by both the Army and Baxter (Estep *et al*., 1989) to remove unreacted hemoglobin. The reaction mixture was heated to 60°C for 10 hours at pH 7.5, and the less stable unreacted tetramers were precipitated out of solution. The final product was formulated in Ringer's acetate for biological testing.

CHARACTERIZATION

Table 33.1 presents a number of properties of the solution produced by the Army. It had approximately 10 g/dl total hemoglobin concentration, and the degree of crosslinking was well above 90 per cent. It was sterile, essentially free of endotoxin, with only trace amounts of phosphate and free iron. The oxygen binding of αα-Hb was described in detail (Vandegriff *et al*., 1989a, 1989b, 1991a, 1998), and showed a *P*50 and degree of cooperativity similar to that of human blood.

The *R*-state oxygen affinity of α-chains is reduced about six-fold, resulting from a decrease in the rate constant for O_2 association and an increase in the constant for dissociation. The

α chain dissociation rate was found to be increased two-fold. The constraints caused by the intersubunit crosslinking are expressed as a five-fold higher rate for the unliganded *R* to *T* allosteric transition. Interestingly, these detailed studies were not repeated for the heat-treated protein, even though subtle alterations in the O_2 equilibrium curves were reported by Baxter (Estep *et al.*, 1988).

Only one measurement of the O_2 equilibrium curve of αα-Hb under physiologic conditions (i.e., PCO_2 40 mmHg, pH 7.4) was reported in the literature (MacDonald and Winslow, 1992). There is no convenient method to measure CO_2 binding to hemoglobin, even though this measurement was recommended by the FDA in its 'Points to consider' document (Center for Biologics Research and Evaluation, 1991). The measurements require large amounts of protein, but Vandegriff and coworkers were able to make them in the laboratory of Dr Michele Perrella in Milan (Vandegriff *et al.*, 1991b). They found that only half of the expected CO_2 saturation was possible in both the liganded (oxy) and unliganded (deoxy) state. They attributed this effect to absent binding of CO_2 at the α-subunit NH_2 termini. Although the importance of CO_2 binding has been raised (Winslow, 1996), no systematic studies on this point have been published.

Nitric oxide binding was measured using the stopped-flow technique by Rohlfs and his colleagues (Rohlfs *et al.*, 1997, 1998). They found that the association rate constants for NO binding to hemoglobin A_0, αα-Hb and bovine hemoglobin surface-modified with polyethyleneglycol (BvHb-PEG5K10) are identical within experimental error. The interesting aspect of these studies is that BvHb-PEG5K10 is essentially devoid of vasoconstrictor activity, while αα-Hb is very vasoactive. These observations challenged the dogma that simple NO scavenging accounts for the vasoactivity observed for cell-free hemoglobin solutions.

The solution properties of a number of modified hemoglobins were reported by Vandegriff and colleagues (Vandegriff *et al.*, 1997a, 1997b). Some of the results are reported in Table 33.2, which gives radius of gyration and exclusion volume as well as viscosity and colloid osmotic pressure. The radius of gyration of αα-Hb is very close to that of both human serum albumin and hemoglobin A_0. Nevertheless, and in spite of an almost identical molecular weight, αα-Hb has a nearly 50 per cent larger exclusion volume, but still only a fraction of the volume of the much larger PEG-hemoglobin. It is of interest that the viscosity of αα-Hb is very low. Its oncotic pressure (COP) is essentially the same as human plasma.

MacDonald and coworkers studied oxidation rates of various modified hemoglobins (MacDonald *et al.*, 1991), and found, in general, that the rates induced by oxidizing agents correlated closely with oxygen affinity, while intramolecular crosslinking decreases the propensity of hemoglobin to precipitate in response to agents

Table 33.1 Some characteristics of αα-Hb produced at LAIR

Quantity (units)	αα-Hb
Osmolarity (mOsm)	285
Hemoglobin (g/dl)	9.95
Methemoglobin (%)	3.2
Total yield (%)	58
pH	7.39
Sterility	Pass
Rabbit pyrogen test (% pass)	100
Total phosphate (ppm)	0.75
Phospholipid (ppm)	<1
Free iron (μg/ml)	4.57
Degree of crosslinking (%)	>90
*P*50 (mmHg)	30
Endotoxin (EU/ml)	0.1–0.2
Buffer	Acetated Ringer's solution

Table 33.2 Physical properties of modified and unmodified hemoglobin

Product	Concentration (g/dl)	Exclusion volume (nm^3)	Viscosity (cPs)	COP (mmHg)	1 R_G (nm)
Hb A_0	7.0	660	1.09	22.6	2.7
αα-Hb	7.9	950	1.00	23	3.1
o-R-Poly-Hb	9.4	4000	1.36	22.9	4.9
HBvHb-PEG5K10	5.5	94 000	3.39	118	14.1

such as menadione and 2-propanol. They warned that any modification that reduces oxygen affinity would likely increase intrinsic rates of methemoglobin formation, which could promote oxidative effects. The rates of heme-globin dissociation were measured by Vandegriff and LeTellier (1994), who found that the rate for αα-Hb was somewhat less than that for hemoglobin A_0, reflecting its greater stability.

PHARMACOKINETICS

One of the earliest studies of plasma kinetics of αα-Hb was carried out by Hess and coworkers (Hess *et al.*, 1989), who reported that the elimination pattern from the plasma fits a two-compartment model with half-lives of 0.7 hours for 20 per cent of the material, and 4.5 hours for the remaining 80 per cent. Under the same conditions, a single compartment model with a half-life of 0.9 hours eliminated hemoglobin A_0. Furthermore, the elimination pattern was not only species-specific, but also dose-dependent. As Table 33.3 shows, there is a weak correlation between the retention time ($T_{1/2}$) and the size of the animal, with monkeys having the longest persistence and rats the shortest. However, in all species there was a striking dependence of $T_{1/2}$ on administered dose. The authors of this study concluded that multiple pathways of clearance from the circulation must operate, and that the pharmacokinetic behavior in man would be difficult to predict on the basis of animal studies.

Although Hess *et al.* (1989) found less than 1 per cent of the administered dose in the urine of their rats, a more extensive study by Keipert *et al.* (1993) found up to 5 per cent of administered αα-Hb in the urine after 24 hours, when administered as a 50 per cent blood exchange. Using a ^{14}C label of the DBBF crosslinker, they showed elimination of 20 per cent of the administered label during the 24 hours after administration. This study, and an earlier similar one (Keipert *et al.*, 1992) concluded that catabolism of administered αα-Hb begins immediately after dosing, and that the kidneys are responsible for eliminating at least the smaller degradation products. A subsequent study by a different group (Urbaitis *et al.*, 1990) also found that 1 per cent of administered αα-Hb was excreted in 24 hours in the rat and that no decrease in Glomerular filtration rate (GFR) was observed. Indeed, both the Urbaitis and Keipert studies demonstrated increases in urine output after dosing and speculated that this was due to sudden expansion of the vascular volume due to oncotic effects. Subsequent measurement of intravascular volume showed the opposite effect, contraction (Migita *et al.*, 1997), and perhaps this should have alerted workers in the field that all the renal effects of αα-Hb had not yet been delineated.

Tissue distribution and long-term elimination studies of αα-Hb (Keipert *et al.*, 1994; see Figure 33.2) demonstrated that about 60 per cent of an administered dose was excreted in the urine, another 10 per cent in the feces, and the balance was unaccounted for. Even though the product had a half-life in rats of 4.5 hours, peak tissue levels were reached in 24 hours, with the distribution shown in Figure 33.2. The widespread tissue distribution suggests that the product must leave the circulation by extravasation, rather than by specific metabolism by the reticuloendothelial (RE) cells, as is the case for red blood cells.

Table 33.3 Plasma elimination of αα-Hb; all hemoglobins were administered as 14-g/dl solutions

Species	n	Dose(ml/kg)	*$T_{1/2}$ (hr)
Rat	6	7	4.4
Rat	6	30	24.0
Rabbit	8	7	12.5
Monkey	6	7	16.0
Pig	1	7	6.8
Pig	1	21	12.4
Pig	1	35	21.7

*$T_{1/2}$ was determined by least-squares fitting to a single compartment model.

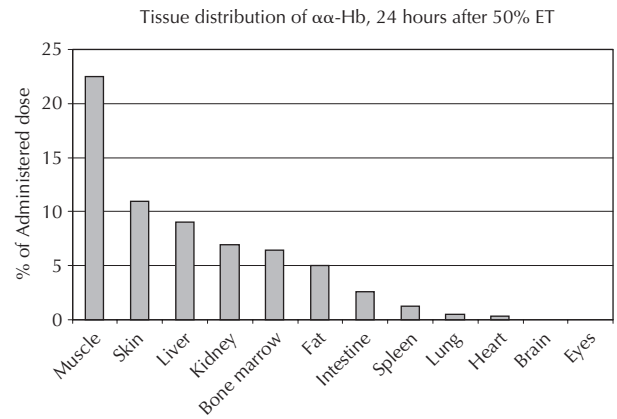


Figure 33.2 Tissue distribution of a dose of ^{14}C-labelled αα-Hb in rats (from Keipert *et al.*, 1994).

VASOCONSTRICTION

As early as 1920, Bayliss noted that hemoglobin solutions increased the blood pressure in rats (Bayliss, 1920), and Amberson noted the same in humans (Amberson *et al.*, 1949). In recent literature on the subject of hemoglobin-induced vasoactivity, there is a tendency to assume that all hemoglobins induce hypertension because of their ability to tightly bind NO, the endothelium-derived relaxing factor (EDRF). Some of the early studies of vasoconstriction were confusing because purified, well-characterized solutions were not yet available. For example, storage of samples seemed to exacerbate the effect (Lieberthal *et al.*, 1987), early preparations of αα-Hb seemed to be less vasoactive in the isolated rabbit heart (Lieberthal *et al.*, 1989), and there was a suggestion that different species of methemoglobin may modulate the effect (MacDonald *et al.*, 1989).

The US Army performed a series of studies to evaluate the clinical utility of αα-Hb, which ultimately led to abandonment of the blood substitute program. Central to this decision was the conclusion that vasoactivity was a negative property that would not be easily overcome. A protocol was designed which simulated battlefield injury (Hess *et al.*, 1991), in which pigs were first dehydrated to reduce their body weight by approximately 7 per cent to simulate the battlefield environment. Then blood corresponding to 38 per cent of body weight was removed over 1 hour, followed by resuscitation by administration of a fixed volume of a test solution. In the preliminary report of this model, no renal toxicity was observed when the animals were resuscitated with αα-Hb (Hess *et al.*, 1991).

The full evaluation of the Army pig model (Hess *et al.*, 1993) showed severe pulmonary and systemic hypertension, reduced cardiac output and elevated systemic and pulmonary resistance (see Figure 33.3). The conclusion of this study was that the additional oxygen-carrying capacity provided by administration of the hemoglobin solution was completely offset by increased resistance to flow, such that the net oxygen delivery (flow × O_2 content) remained the same. The study concluded that resuscitation with αα-Hb was no better, in terms of O_2 transport, than resuscitation with either Ringer's lactate or a 5% albumin solution. A variety of studies was carried out at LAIR following these observations which were aimed at understanding the many cellular, biochemical and physiological effects of αα-Hb, as well as improvements in production. These studies implicated the roles of NO scavenging and free radical mechanisms (Motterlini and MacDonald, 1993; MacDonald and Motterlini, 1994), but no clear unifying hypothesis to explain the lack of efficacy in the pig model emerged.

In rats, administration of αα-Hb produces two hallmark effects: an immediate rise in mean arterial pressure, which is not dose-dependent at doses above 62.5 mg/kg (Malcolm *et al.*, 1992) and a reciprocal drop in heart rate (Figure 33.4). Thus, vascular resistance is elevated, since resistance is pressure/cardiac output. This suggests a mechanism or mechanisms that saturate at relatively

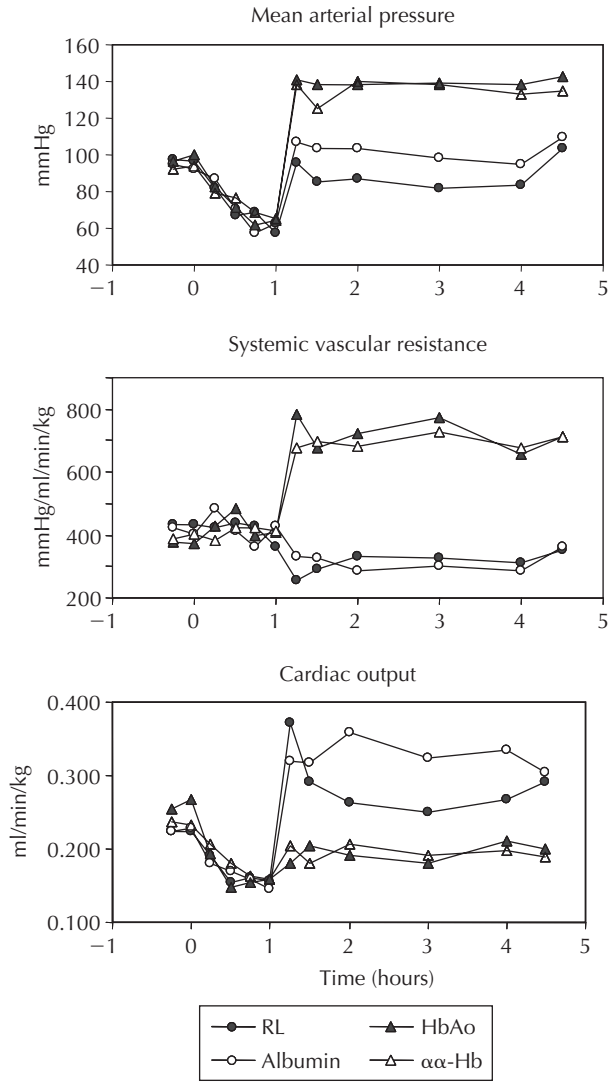
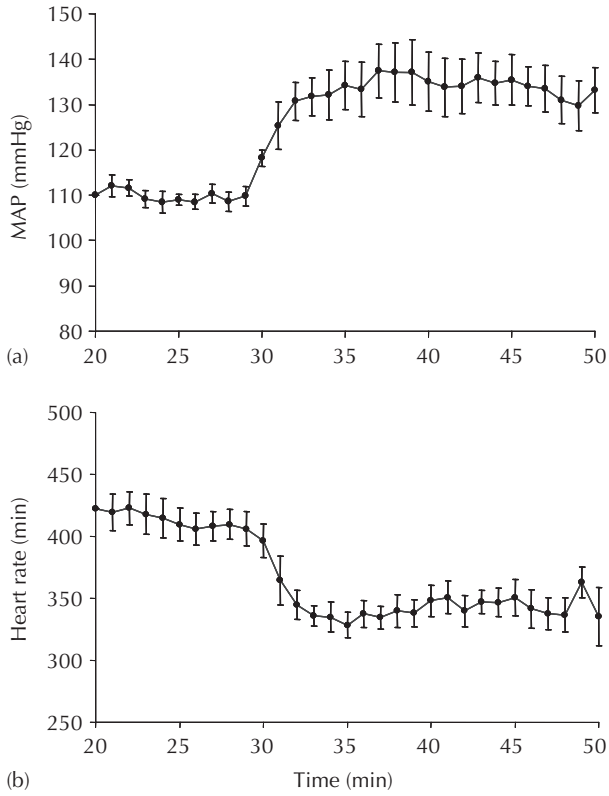


Figure 33.3 Resuscitation of dehydrated pigs with αα-Hb in the US Army model of battlefield injury (modified from Hess *et al.*, 1993).



low hemoglobin concentrations. Neither effect can be a result of volume changes, but rather suggest a direct pharmacological effect (Migita *et al.*, 1997). Studies using the Army's αα-Hb showed that the hypertensive effect could be ameliorated somewhat by polymerization, increasing its molecular volume (Abassi *et al.*, 1997). Hemoglobin molecules that are still larger, such as PEG-hemoglobin, produce even less vasoconstriction (Winslow *et al.*, 1998).

HEMODYNAMICS

Elevation of blood pressure, especially in hypotensive conditions such as hemorrhagic shock, is not necessarily bad. However, elevated pressure is only clinically or physiologically useful if it is accompanied by increased blood flow. For example, tissue perfusion can often be improved in shock patients when pharmacological agents are given that increase cardiac output primarily by affecting the heart rate (chronotropic agents) or the force of cardiac contraction (inotropic agents). In distinction from these mechanisms, agents that elevate blood pressure by peripheral vasoconstriction do so at the expense of raising peripheral resistance, so that capillary blood flow may decrease.

Since hemoglobin is a colloid, like albumin, it has the potential to expand and retain vascular volume. However, the COP (colloidal osmotic pressure) of αα-Hb is relatively low, so this is not expected to be a pronounced effect. Measurements of the blood volume were made following administration of αα-Hb to two groups, one after a 50 per cent (of estimated blood volume) exchange (Migita *et al.*, 1997) and the other after a top load of approximately 30 per cent of estimated blood volume (Fischer *et al.*, 1999). The two protocols were therefore different, and came to different conclusions. In the exchange protocol the blood volume was seen to contract following administration, while it expanded somewhat after top-load infusion. The former study, intended to be an isovolemic exchange, indicated that as the low molecular weight αα-Hb is cleared from the circulation it carries water with it, leading to a net reduction in plasma volume. In retrospect, this study might have been a warning that unless sufficient volumes of intravenous solutions are given, administration of αα-Hb could actually lead to depletion of the vascular volume. To the extent that the loss of αα-Hb is a reflection of renal filtration and elimination, it could indicate a net dehydrating effect, which is not a desired outcome in resuscitation from acute blood loss.

MICROCIRCULATION

As mentioned above, blood pressure can rise by either of two mechanisms: increased cardiac work, or peripheral vasoconstriction. The only way to discern between the two is to visualize the microvessels directly. Methods such as the distribution of colored or radioactive microspheres may be tissue-specific, but provide no information about whether or not small microvessels, including capillaries, are perfused.

When red cells are progressively removed, as in hemodilution or hemorrhage, vascular beds react by constriction, raising peripheral resistance, lowering peripheral flow, and preserving central volume and cardiac output. These changes can be directly observed in some vascular beds, such as

the hamster skinfold, cheek pouch, and various mesentery preparations. Tsai and coworkers (1995) quantitated these changes when the hemodiluent was αα-Hb, using Dextran-70 as a control. They reported that when the hematocrit was reduced to about 10 per cent, the number of capillaries that contain flowing red cells ('functional capillary density', FCD) was reduced to about 50 per cent of control levels, while in the dextran animals it was reduced to only about 80 per cent of baseline. Calculation of O_2 transport in these animals showed that in spite of the addition of the O_2 carrier, O_2 available to tissues was offset by decreased tissue perfusion. Notably, this is exactly the same result as observed by the US Army in the pig studies cited above.

Microcirculation studies using less severe hemodilution did not agree with those of Tsai *et al.*, but the reduction in tissue perfusion with αα-Hb is not observed until the degree of hemodilution is severe. Thus, experiments in animals under relatively mild conditions (nonlethal) may not be expected to show this undesirable effect of αα-Hb. As an example, a study of resuscitation from hemorrhagic shock by Kerger *et al.* (1997) showed that animals that survived a severe shock insult had significantly increased functional capillary density when compared to animals that went on to die.

The microcirculation studies by the Intaglietta group led to a general hypothesis regarding the role of viscosity in tissue oxygenation (Tsai *et al.*, 1997). Using a mass balance mathematical model of oxygen transfer in the microvessels, this theory suggests that maintenance of blood viscosity is essential to normal endothelial function, and when viscosity falls significantly below the normal value (4 cPs) elaboration of natural vasodilators, NO and prostacyclin may be sharply reduced, leading to vasoconstriction. This proposal has been recently confirmed by studies of hemodilution with dextrans of various viscosities (Tsai *et al.*, 1998). Furthermore, increased viscosity (and molecular size) is expected to reduce 'facilitated diffusion' of cell-free hemoglobin (Bouwer *et al.*, 1997; Nishide *et al.*, 1997), which can itself lead to vasoconstriction by autoregulatory mechanisms (Winslow *et al.*, 1998). Increased viscosity of cell-free hemoglobin solutions is a counterintuitive approach, but the concept that lowered viscosity might lead to improved perfusion is based on analogy with red cells suspended in plasma, not a cell-free oxygen carrier.

Confirmation that the mechanism of hypertension in the case of αα-Hb is peripheral vasoconstriction was provided in a study of hemodilution and consequent hemorrhage in rats (Winslow *et al.*, 1998). In this study, where 50 per cent of the circulating blood volume was replaced with αα-Hb, cardiac output fell while pressure and resistance rose, clearly indicating marked peripheral vasoconstriction. This is clearly not what is wanted in shock resuscitation, where patients are already peripherally vasoconstricted. In fact, the opposite is desired: vasorelaxation and greater cardiac output.

ENDOTHELIUM

The endothelium is an extremely important but not perfectly understood organ. Endothelial cells carry out considerable work, consuming large amounts of oxygen. In turn, these cells carry out a wide variety of functions, including synthesis of coagulation factors as well as multiple peptides, some with vasoactive properties. Endothelial cells are also the site of synthesis of NO, which has been identified as the endothelium-derived relaxing factor (EDRF). The metabolic activity of endothelial cells may also be regulated by local shear stress, which is viscosity dependent (Ballermann *et al.*, 1998). Therefore, understanding the biology of endothelial cells during red cell replacement with cell-free oxygen carriers is essential.

Few studies of direct measurement of endothelial leakage have been published. However, Baldwin has observed that αα-Hb and BvHb-PEG5K10 (Enzon) can both extravasate by a mechanism that includes widening of interendothelial gap junctions in the rat mesentery (Baldwin, 1997, 1999; Baldwin *et al.*, 1998). In these studies, while both model hemoglobins induced venular leakage, the effect of αα-Hb was much greater than that of BvHv-PEG5K10. The latter is a much larger molecule, with a much higher viscosity (Vandegriff *et al.*, 1997a).

When αα-Hb is infused into animals, oxidation to methemoglobin occurs at a slow and constant rate, accompanied by release of heme, which is taken up by endothelial cells. Motterlini *et al.* (1995a) demonstrated the induction of heme oxidase in cultured endothelial cells and cell injury. This group concluded that the higher rate of autoxidation of αα-Hb, relative to the rate for unmodified hemoglobin, is responsible for

this heme release (Motterlini *et al.*, 1995b). They regarded heme oxidase induction to be a protective mechanism.

OXYGEN TRANSPORT

Oxygen transport is often considered to be equivalent to *P*50, or the position of the oxygen equilibrium curve. This is certainly not the case, and particularly not when dealing with cell-free hemoglobins. This complex issue has been discussed in detail in theoretical papers (Vandegriff and Winslow, 1995; Winslow and Vandegriff, 1997), which have suggested that increased rather than decreased oxygen affinity may actually contribute to better tissue oxygenation. Experimental evidence has been presented to suggest that hemoglobin, free in the intravascular space, should increase tissue oxygenation (Federspiel and Popel, 1986; Lemon *et al.,* 1987; Page *et al.,* 1996, 1998; Boland *et al.*, 1997). However, Hogan *et al.* (1994) were unable to show the effect in dogs using $\alpha\alpha$-Hb, and the same lack of effect was noted by Biro *et al.* (1991) for polymerized hemoglobin. These data were key in formulating the theory of the 'oversupply' of O_2 by cell-free hemoglobin as contributing to autoregulatory vasoconstriction (Winslow and Vandegriff, 1997).

OXIDATION

Outside of the red blood cell, enzymes like hemoglobin reductase and superoxode dismutase no longer protect hemoglobin. By virtue of its iron content, hemoglobin can participate in a number of reactions that could potentially produce toxic agents (Everse and Hsia, 1997), and various authors have generalized the concern to condemn all hemoglobin products (Eaton, 1996). Reaction with small amounts of H_2O_2 can produce the highly reactive hydroxyl radical ($^{\bullet}OH$) and the ferryl iron species (Hb^{4+}), which is a strong oxidant (Alayash, 2000). When the iron oxidizes to form methemoglobin ($HbFe^{3+}$), a number of reactions can occur, including reaction with NO (Alayash *et al.*, 1999), reducing this natural vasodilator and leading to vasoconstriction. Alayash has pointed out that these reactions could be especially toxic in the reperfusion setting, where another redox form, peroxynitrite ($ONOO^{-}$) forms, which can decompose to hydroxyl radicals.

Alayash and coworkers carried out a series of studies showing that hemoglobins, crosslinked in various ways, differ in their reactivities with H_2O_2. These studies showed that the tendency to form the ferryl species in the presence of H_2O_2 was different for $\alpha\alpha$-Hb compared to unmodified hemoglobin A_0 (Cashon and Alayash, 1995; D'Agnillo and Alayash, 1999). They subsequently showed that generation of free radical species by $\alpha\alpha$-Hb was less than the rate for unmodified HbA_0, and independent of oxygen affinity (Alayash *et al.*, 1992). The studies reported by these workers used the $\alpha\alpha$-Hb obtained from the US Army.

From a biochemical point of view, these studies show that generation of oxygen-derived free radicals is *possible* when cell-free hemoglobin is present in the plasma. Furthermore, the tendency to form such radicals can be modulated by the specific chemical modification. Studies on cultured endothelial cells (Goldman *et al.*, 1998) have shown that hemoglobins, including $\alpha\alpha$-Hb, can accelerate programmed cell death (apoptosis), correlating with the formation of ferryl hemoglobin derivatives. However, there is as yet no link between these biochemical phenomena and the physiological or clinical effects of the solutions. In order to establish such a link, some measurement would be required to quantitate the formation of free radicals in animals or patients to whom such products are given. The concerns over the oxidant properties of cell-free hemoglobin were recently summarized by Alayash (Alayash, 1999).

CENTRAL NERVOUS SYSTEM

One of the first concerns of the US Army laboratory was over preliminary studies, which indicated that after administration of cell-free hemoglobin to rats, their ability to negotiate a learned rat-maze was diminished (unpublished data). Therefore, when relatively pure $\alpha\alpha$-Hb became available, the studies were carefully repeated (Przybelski *et al.*, 1989, 1990). These and other studies of general behavior (Bauman *et al.*, 1991) failed to show any detrimental effect, and the earlier results were attributed to impure or poorly characterized hemoglobin solutions.

The LAIR group (Panter *et al.*, 1994), studied the effects of $\alpha\alpha$-Hb on rat cortical cell cultures. The hemoglobin was found to be neurotoxic in these cultures, and the effects were dose-dependent and reversible by administration of

an antioxidant or an iron chelator, desferrioxamine. That this is a hemoglobin effect was confirmed by the blockage of the toxicity by the coadministration of the hemoglobin-binding protein, haptoglobin.

EDITOR'S SUMMARY

In 1998, Baxter Healthcare announced that it was abandoning its product, DCLHb, the first 'blood substitute' to complete all phases of human trials. The company announced that the Phase III (pivotal) trials in humans had resulted in an unexpectedly high survival in a group of patients serving as controls for those who received their product in a trauma setting. It is not possible to quantitate the time, effort and money that were expended in the course of developing this product from 1985 to 1998. It is rumored that the giant healthcare company had expended more than half a billion dollars on this product, not to mention the investment in the same product by the US Army, the National Institutes of Health and many independent university-based scientists. The disappointment was profound and far-reaching. Although the threat of HIV transmission by banked blood has all but disappeared in the developed world, still the bulk of the world's population faces blood shortages, which this product and its future generations might have helped alleviate. Only Baxter and the FDA may forever know key elements of the history of development of this product. However, because the US Army decided to make its version of the product widely available to scientists, there is a substantial published record, contributed to by both Baxter and independent scientists. Examination of this record leads to the conclusion that there is no single reason for failure. However, it shows that the characteristic hemodynamic response caused by αα-Hb, increased vascular resistance, probably eliminates its potential as a red cell substitute. Newer solutions that overcome this limitation should fare better in clinical development when this problem is overcome.

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DCLHb and rHb1.1

Chapter 34

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'TETRAMERIC' MODIFIED HEMOGLOBINS

Two intramolecularly crosslinked hemoglobin tetramers – DCLHb and rHb1.1 – stand out from among those in a cornucopia of hemoglobin derivatives that were prepared at laboratory scales and screened in the late twentieth century. Each of these proteins was designed to meet then-current requirements for 'blood substitutes'. For example, it was widely postulated that each would be a *replacement* for transfusion of packed red cells, and provide *both* colloidal volume expansion *and* oxygen-delivery characteristics similar to those exhibited by fresh whole blood. Likewise, stringent purification and incorporation of an internal crosslink to prevent dissociation of cell-free hemoglobin into dimers were goals that would, it was believed, address still other shortcomings of unmodified acellular hemoglobin.

At the time, preparation of a stabilized Hb tetramer modeled after human Hb appeared to be reasonable and sufficient to meet the postulated requirements for an 'artificial blood'. It was believed that a tetramer would be sufficiently large to lengthen the circulating half-life and prevent the hemoglobinuria and renal injury that restricted use of unmodified acellular hemoglobin. In addition, advances in protein characterization had pinpointed ways in which dissociation to dimers could potentially be eliminated by incorporation of inter-subunit crosslinks, either embedded in the globin or at termini of the α-subunits, which lay in close proximity.

Likewise, prevalent assumptions concerning the optimal oxygen-binding characteristics of an acellular hemoglobin guided blood substitute development. Within the human red cell, it was recognized, oxygen release is facilitated by the binding of the polyanionic allosteric effector, 2,3-diphosphoglycerate (2,3-DPG), which stabilizes hemoglobin in the lower oxygen affinity conformation. During red cell storage, the concentration of 2,3-DPG decreases to near undetectable values, and the hemoglobin in the stored RBCs has a higher affinity for oxygen. As a consequence of the significant increase in oxygen affinity, marked by a left shift in the oxygen dissociation curve, oxygen delivery decreases from ~4.7 g/dl immediately after blood collection to less than half that value about 10 days later, when the 2,3-DPG is fully depleted. These observations suggested that the oxygen-delivery characteristics of choice would comprise a $P50$ of about 28 mmHg (with a Hill coefficient, n, of about 2.9) and an oxygen-delivery capability of about 5 ml/dl.

To achieve these goals, chemistry or biotechnology, respectively, was developed to ensure highly specific intramolecular crosslinking. In the case of DCLHb, application of the crosslinking technology concurrently accomplished favorable alteration

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of the oxygen-binding function. In the case of the recombinant hemoglobins, an Asparagine 108β → Lysine mutation (i.e., the Hb Presbyterian mutation) altered the oxygen-binding function in a manner that mimicked that of fresh blood.

DCLHb and the HemAssist™ (DCLHb 10 per cent and electrolyte injection, Baxter Healthcare Corporation) dosage form in which it was incorporated are unique among other hemoglobin-based oxygen carriers (HBOCs) of the period in many respects. For example, these products provided to the clinical community consistent, injectable-quality material that was produced from human hemoglobin using rugged and reproducible, validated processes at three different manufacturing sites, including one in Europe. These properties encouraged investigators both to probe more exhaustively and to expand their research into the properties and potential therapeutic applications of acellular hemoglobin. The resulting studies of DCLHb confirmed that, in addition to its oxygen-carrying properties, Hb functions as an active oncotic and pharmacological agent. Ultimately, these same properties also contributed to its demise for applications as anything other than a research tool.

Similarly, the successful development of recombinant hemoglobin and its injectable dosage form, (Optro™, recombinant human hemoglobin 8 g/dl and electrolyte injection, Somatogen, Inc.) marks a unique achievement. The ability to prepare cell-free hemoglobin solutions using human hemoglobin synthesized in *E. coli* and the yeast *Saccharomyces cerevisiae* presented an attractive alternative to blood substitutes based on hemoglobins sourced from mammalian red cells. Further, the observation that a recombinant Hb could be modified by the techniques of protein engineering to modulate oxygen affinity and stabilize the protein from dissociation ensured a consistent supply of an oxygen-delivering protein free of blood-borne pathogens.

At the time, it was widely recognized that, in many respects, *E. coli* is an excellent choice of organism in which to express heteromultimeric proteins such as human hemoglobin. Nonetheless, Somatogen's decision in the early 1990s to continue development in *E. coli* of a recombinant hemoglobin for use as a blood substitute corresponds to near-swashbuckling bravado that, even today, flies in the face of prevailing wisdom. First and foremost, investigators had repeatedly warned against the toxicities associated with interactions between bacterial endotoxin and hemoglobin (Levin and Roth, 1998). Expression in *E. coli* provided molecules of recombinant hemoglobin awash in a sea of bacterial endotoxin. In addition, fermentation substituted a source containing about 95 per cent of the desired hemoglobin raw material with a source containing, at best, about 15 per cent of the desired raw material in a complex mixture of other proteins (Bunn and Forget, 1986). The ability to scale rHb fermentation and processing to commercial volumes in a reproducible manner crowns the rHb team's achievements.

The systematic manipulation of the physicochemical properties of recombinant hemoglobins also distinguishes the rHbs from other HBOCs of the period. In particular, design and production of a polymerized rHb having a significantly reduced rate of reaction with nitric oxide and acceptable oxygen-binding properties positioned rHb2.0 for imminent clinical success. Unfortunately, the observation of an unexpected and unexplained side effect in Phase I clinical trials of these novel rHbs devastated the program and metamorphosed recombinant hemoglobins into research tools.

DCLHb

Composition

The term DCLHb, an acronym for diaspirin crosslinked Hb, refers to a modified human hemoglobin that is stabilized as a tetrameric protein by a (bis)fumaramide bridge between ε-amino groups of the Lysine 99 amino acid residues of each α–globin chain. The covalent bridge is formed by reaction of purified human hemoglobin with bis(3,5-dibromosalicyl) fumarate (DBBF) under reaction conditions (described below) that were developed by Baxter Healthcare Corporation (Hai *et al.*, 1992) from laboratory-scale preparations of Walder *et al.* (Chatterjee *et al.*, 1986) The covalent bis(fumaramide) crosslink is chemically stable, as evidenced by the observation that the size-exclusion chromatographic profile of DCLHb does not change after lengthy storage at −20°C or lower temperatures, at refrigeration temperature, at room temperature, intravascularly after infusion, and even after metabolic or hydrolytic degradation of the protein. The name DCLHb is inaccurate, since there is no 'aspirin' in the crosslink (fumarate) or the modified hemoglobins, but over the years it has been widely used in the scientific literature.

The trade name for clinical formulations of DCLHb was HemAssist™ (DCLHb 10 per cent and electrolyte injection). HemAssist consisted of a 10-g/dl solution of DCLHb in a balanced electrolyte solution, iso-osmotic with whole blood and having a pH of 7.4 at 37°C. The balanced electrolyte vehicle contained the major ions (sodium, potassium, calcium, magnesium and chloride) found in human plasma in their normal serum concentrations. Bicarbonate, a major constituent in plasma, was replaced in the electrolyte formulation by lactate, a bicarbonate precursor. This substitution was reasonable, since lactate is a constituent of normal human plasma, albeit at lower concentrations than bicarbonate. The substitution of lactate for bicarbonate also addressed three potential stability issues related to bicarbonate. First, at physiological pH, the bicarbonate–carbon dioxide equilibrium permits outgassing of carbon dioxide and subsequent increases in solution pH with time. The pH of lactate-containing formulations was stable. Second, in electrolyte formulations at physiological pH, calcium may be precipitated by (bi)carbonate. Calcium is not precipitated by lactate under these conditions. Finally, lactate was one of several α-hydroxyacids that slowed the rate of DCLHb oxidation and protected the protein against damage during solution freezing.

The HemAssist formulation also addressed a number of perceived user requirements. For example, this dark red, oxygenated, aqueous solution had a viscosity similar to that of water. At a concentration of 10 g/dl of DCLHb, the oxygen-carrying capacity of HemAssist was approximately three-fourths that of normal human blood (i.e., blood having an average total hemoglobin concentration of 13–14 g/dl) and the oncotic pressure was about 50 per cent higher than that of normal human blood (about 42–44 mmHg for HemAssist versus an average of 27 mmHg for human plasma). The elevated oncotic pressure was believed to be an advantage in the treatment of shock states and the alleviation of tissue edema.

Likewise, the development teams recognized that ease of use is a key factor in clinical acceptance of a new product. (The first commercial perfluorocarbon emulsion to be used in clinical trials, Fluosol-DA® (Green Cross Corporation, Osaka, Japan), was approved in 1990 in the US and Britain for oxygenating the heart during angioplasty, but its acceptance was limited by the complexity of its preparation for infusion, and its use rapidly declined because of poor efficacy and short persistence in the bloodstream, together with concerns about the hepatotoxicity of the emulsifying agent that was used in the formulation. The product was voluntarily withdrawn from the marketplace in 1996.) A variety of product configurations were considered as part of HBOC product development. No technical barriers prevented development of oxygenated, deoxygenated, or lyophilized dosage forms (although unfortunately the chasm separating available technology and product requirements prevented development of an encapsulated dosage form). Lyophilized dosage forms appeared to be the most difficult and limiting of these options, both in terms of formulation and processing and with respect to complexity of user preparation (admixture) prior to infusion. Oxygenated HBOCs could be infused immediately, but increases in methemoglobin content (which is in general the key stability-indicating parameter in these carriers) limited storage at room temperature to a few hours and at refrigerated temperatures to several days. (If appropriately formulated, HBOCs could be stored at −20°C for more than a year and at −80°C for several years.) DeoxyHBOCs could also be infused immediately (deoxyHb reoxygenates rapidly on exposure to air) and had excellent storage stability at room or refrigerated temperatures, but these positive attributes were coupled with a stringent requirement for absence of exposure to oxygen until the time of use. Glass containers provided the best oxygen barriers, but presented weight and disposal issues. In the end, flexible containers constituted the packaging configuration of choice. DCLHb was packaged in ethylene-vinyl acetate (EVA) containers and stored frozen. The DCLHb team clearly showed that poly(vinyl chloride) containers are inappropriate packaging materials for proteins, as the plasticizer is extracted into the infusate.

Manufacture

A general scheme for the manufacture of DCLHb and HemAssist, the trade name for the potential product containing this biologic, is provided in Figure 34.1.

The source of hemoglobin for use in the manufacture of DCLHb was human red blood cells (RBCs) that were collected from volunteer donors by licensed or registered blood centers, tested, and found non-reactive for human immunodeficiency virus, hepatitis B virus and hepatitis C virus (HIV, HBV and HCV, respectively). If the packed RBC

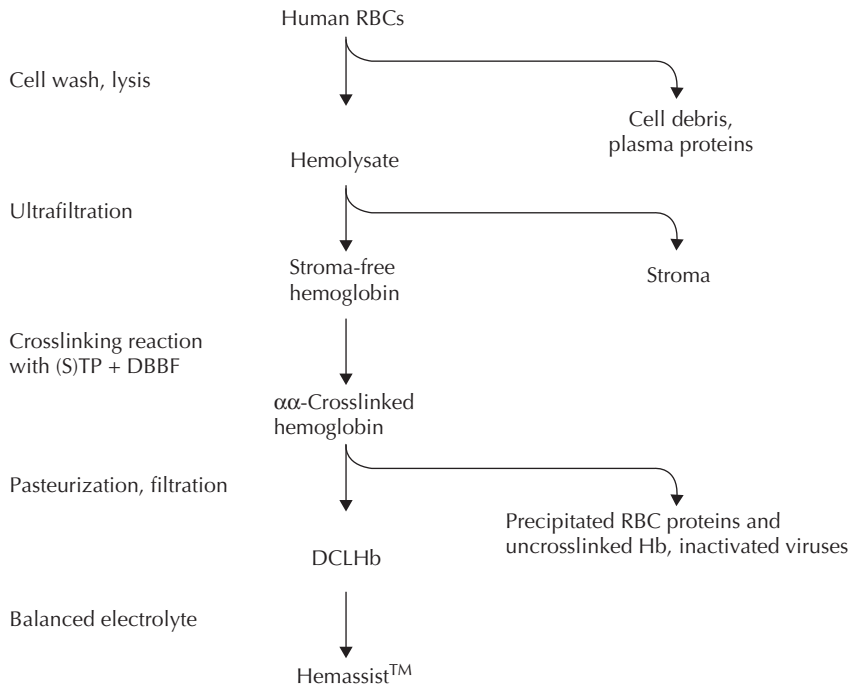

Figure 34.1 Schematic diagram of the HemAssist™ manufacturing process.

units were suitable for transfusion, they could be included in the raw material pool, irrespective of whether they were in-date or expired. (Large-scale extraction and purification of hemoglobin can be efficiently performed even after the red cell units have exceeded their normal shelf life.) Theoretically, RBCs of any blood type could be used; practically, Type A and B units were most readily available in bulk quantities from blood centers.

Human erythrocytes were selected as the preferred hemoglobin source because there is less potential for human-sourced material to cause adverse immune responses in human recipients as compared to that of hemoglobin derived from other sources. In addition, a vast amount of data has been amassed concerning the chemical and physical properties of human red cells. Although there is a low but finite risk of disease transmission upon infusion of human blood, this risk is well understood compared with the disease risks associated with blood from other sources. The risk of disease transmission was further minimized during processing, when validated ultrafiltration and virus inactivation steps were used to eliminate adventitious contamination.

The DCLHb production process began when RBCs were pooled aseptically and then washed (diafiltered) with slightly hypertonic saline solution to reduce the concentration of extracellular proteins. The washed cells were lysed by exposure to hypotonic phosphate solution to release hemoglobin, and the hemolysate was ultrafiltered with water to produce stroma-free hemoglobin. The purified, stroma-free hemoglobin could be prepared as sterile bulk and stored under refrigeration or used immediately.

Crosslinking was accomplished by deoxygenating stroma-free Hb in the presence of a 2,3-DPG analog (tripolyphosphate) and allowing the deoxyHb thus obtained to react with DBBF (added in slight molar excess as a solution in HEPES buffer). Because the 'central water cavity' is open when Hb is in its deoxy conformation and the other preferred site for crosslinking between the β-chains was blocked by tripolyphosphate, crosslinking introduced a fumaramide bridge between the terminal amino groups of the two lysine-99α residues with remarkable specificity. (Crosslinking with DBBF corresponds to nucleophilic displacement of a good leaving group from an activated ester and does not cleave existing covalent (peptide) bonds.) The crosslinking reaction was monitored and when the extent of crosslinking reached a plateau, covalently crosslinked, stabilized hemoglobin tetramer was the major Hb present in solution.

Purification included both a pasteurization step and ultrafiltration. Pasteurization was performed following crosslinking to assure virus inactivation as well as denaturation and precipitation of non-crosslinked hemoglobin and other heat-labile proteins. Then, for the first time since crosslinking was initiated, the crude product mixture was reoxygenated and ultrafiltered to remove denatured protein particulate. To achieve physiological compatibility, the final DCLHb solution was diafiltered in step-wise fashion into a balanced electrolyte solution, and the solution pH was adjusted to physiological values. The final product was then filter-sterilized and aseptically filled into individual EVA containers. The units were frozen at −20°C or lower temperatures to enhance long-term stability (oxidation of DCLHb to the inactive methemoglobin form was found to be the stability-limiting degradation process; methemoglobin content increased at an average rate of about 0.3 per cent per month during frozen storage). Overall yields from commercial processing ranged from 50–60 per cent, based on unmodified hemoglobin.

Batches of HemAssist were required to pass more than 30 validated tests and assays prior to release. In addition to widely used characterizations for HBOCs (Table 34.1), more than a dozen additional tests were performed to evaluate the quality of the product (Nelson *et al.*, 1992a; Azari *et al.*, 1994).

Table 34.1 Tests and assays performed as part of GMP release of HemAssist™ (the tests and assays on the left were product specifications and those on the right provided additional information about each batch)

Release Tests	Other Tests
Total hemoglobin	Filterability
Methemoglobin	Particulate/30 ml
pH at 37°C	Albumin (ppm)
*P*50 (cooperativity)	IgG (ppm)
Per cent crosslinking	Aggregates
General safety	Acetate
Sterility	Isoelectric focusing
Pyrogenicity	SDS-Page
LAL	RP-HPLC
Rabbit	
Excipients	
Sodium	
Potassium	
Magnesium	
Chloride	
DL-Lactate	
Calcium	
Osmolality	
Process residuals	
3,5-Dibromosalicylate	
Residual phosphate	
HEPES	
Phospholipids	
Fumarate	

Physicochemical characteristics of DCLHb

DCLHb (Chemical Abstracts Service Registry Number 142261-03-8) comprises a microheterogeneous family of stable, covalently crosslinked hemoglobin derivatives. The microheterogeneity exhibited by DCLHb derives first from the fact that human hemoglobin is a microheterogeneous protein, and secondly from the observation that reaction with DBBF yields crosslinked hemoglobins that may be modified at other sites on hemoglobin in addition to the predominate reaction with Lysine 99 residues. Each member of the DCLHb family shares a number of characteristics. Thus, the amino acid sequence of the protein subunits of DCLHb is identical to that of the α- and β-subunits of human hemoglobin with the exception that the α-subunits of DCLHb are joined covalently by a fumaramide bridge at Lysine 99α. (The position of crosslinking was confirmed by X-ray crystallography, amino acid analysis and mass spectral structural characterization; see Snyder *et al.*, 1987; Jones, 1989; Fernandez, 1995; Yu *et al.*, 1997.) The UV-visible absorption spectra of DCLHb and native human hemoglobin are superimposable. Likewise, the circular dichroism characteristics of these two proteins are identical, within experimental error.

Size-exclusion chromatography revealed that DCLHb comprises αα-crosslinked hemoglobins having two molecular sizes: about 96–98 per cent of the αα-crosslinked hemoglobins in DCLHb have a molecular weight of about 64 kDa, and 2–3 per cent of the αα-crosslinked hemoglobins in DCLHb have a molecular weight of about 130 kDa. At this same level of sensitivity, a third peak, typically comprising a few tenths of a per cent of the total area response of the test sample, is observed; it is residual non-crosslinked hemoglobin. The molecular weights of both crosslinked proteins were assigned on the basis of relative HPLC retention volume and corollary electrophoretic and mass spectrometric analyses of isolated fractions, all of which confirmed the assignments. Mass spectra indicated that the

130 kDa component of DCLHb comprises *inter*-molecularly crosslinked DCLHb – i.e., dimers of DCLHb tetramers in which a second fumaramide bridge covalently linked one DCLHb tetramer to an $\alpha\alpha$ or β subunit of a second DCLHb tetramer via other lysine residues having surface-exposed amino groups that were available for reaction with DBBF (Yu *et al.*, 1997).

To all intents and purposes, the functionality and structural integrity of native human hemoglobin were maintained in DCLHb. The low oxygen affinity ($P50$ of 32 ± 3 mmHg) and high degree of cooperativity of oxygen binding exhibited by DCLHb solutions were similar to the corresponding properties of hemoglobin in the RBC *in vivo*, and remained unchanged throughout the proposed shelf-life of commercial product. Both the 64.5-kDa and 130-kDa components of DCLHb are capable of reversible oxygen binding and release (Yu *et al.*, 1997). Like the native human hemoglobin from which it was derived, DCLHb also contained two reactive sulfhydryl groups per molecule. As is true of unmodified human hemoglobin, the ferrous iron within the heme groups of DCLHb underwent a gradual oxidation to the ferric state at rates dependent on the solution matrix, the temperature and the partial pressure of oxygen.

Product history

Over time, a broad spectrum of preclinical and clinical studies was completed that suggested strongly that DCLHb was capable of restoring blood pressure and circulation following acute hypotension after insults such as hypovolemic shock. Moreover, preclinical studies suggested that DCLHb might be useful to restore and maintain perfusion of key organs following myocardial infarction, stroke or septicemia (Nelson, 2000, 2002). Other potential applications that were examined included treatments for anemia, head injury, cachexia and sickle cell crisis; enhancing cancer treatments; stimulating hematopoiesis; improving repair of physically damaged tissues; and alleviating cardiogenic shock. Taken together, the data supported pivotal clinical trials evaluating use of DCLHb for reducing or preventing the postoperative use of blood transfusions and as an adjunct therapy for enhancing oxygen delivery in trauma and hemorrhagic shock patients, as well as a prospective study in which DCLHb was administered to stroke patients to mitigate the effects of focal ischemia.

Almost from the date of project inception, the Baxter team recognized that 'next-generation' hemoglobins, likely derived from DCLHb, would be required in order to match a particular clinical need most closely with specific product characteristics. Further, as the program evolved around new knowledge, it was concluded that additional modification of DCLHb had the potential to attenuate effects related to interaction with nitric oxide or the formation of heart lesions. Non-specific crosslinking agents such as glutaraldehyde were studied extensively but provided heterogeneous mixtures of highly modified hemoglobins that generally displayed poor stability, a propensity to autoxidation, and significantly increased oxygen affinities (Hai *et al.*, 1994). In addition, exposure to peripheral blood monocyte cells elicited expression of cytokines (e.g. TNF-α and IL-6), a response that was not seen with DCLHb. Modification with oxyethylene-containing polymers (e.g. poly(ethylene glycol) derivatives, Denacols, and proprietary polyamides; Hai *et al.*, 1994; Nelson *et al.*, 1992b, 1993) showed significantly greater promise, in that extended circulating half-lives as oxyhemoglobin were observed following infusion in animal models and side effects were significantly mitigated (Leppäniemi *et al.*, 1996). Likewise, modification with proprietary chondroitin polymers enabled production of hemoglobins of uniformly increased molecular volumes (Hai *et al.*, 1999); concomitant benefits of a magnitude similar to those of the Denacol-substituted DCLHbs were, unfortunately, not observed.

DCLHb development ended in 1999. Concerns about the ability to manage RBC sourcing to meet ever more stringent regulatory requirements and the long-term availability of this raw material, combined with a tsunami of new knowledge concerning nitric oxide, physiological oxygen delivery and the pharmacological effects of acellular hemoglobin, mired the program in a swamp of rapidly changing, but poorly defined requirements. Adverse findings forced the abrupt termination of active clinical trials (Saxena *et al.*, 1998; Nelson, 2002). The promise of successful development of a recombinant hemoglobin that was carefully engineered to address all the shortcomings of DCLHb tolled the death knell for therapeutic use of this crosslinked hemoglobin. The possibility that DCLHb offers advantages for some niche clinical indication was quashed, and investigators directed attention to genetically engineered mutants and modified recombinant

hemoglobins having larger sizes as better candidates for hemoglobin-based oxygen carriers.

RECOMBINANT HEMOGLOBINS

Composition and nomenclature

Originally, this series of proteins was identified by a naming convention taking the form rHb#.# (Brucker, 2000). The letters 'rHb' indicated that the protein is a recombinant hemoglobin, with an initiator methionine replacing the first residue of each subunit chain, as is typical of a protein expressed by *E. coli*. The first numeric variable was 1 if the Presbyterian mutation (Moo-Penn *et al.*, 1978) was present or 0 if it was absent. The second variable specified the number of glycine residues linking the α subunits of the hemoglobin tetramer, with 0 indicating no fusion. Thus, proteins identified as rHb1.0, rHb1.1, and rHb1.2 refer to recombinant hemoglobins containing the Presbyterian mutation and having no, one or two glycines crosslinking the α subunits, respectively.

As time passed and sizable libraries of mutant Hbs were assembled, the naming convention changed. Baxter elected to identify its 'next-generation' recombinant Hbs numerically by generation. Thus, rHb2.x refers to a second-generation rHb, rHb3.x to a third-generation product, and so forth.

Somatogen's injectable dosage form was trade-named Optro™, and consisted of a sterile, liquid, ready-to-use 8 g/dl rHb1.1 solution in an ascorbate-containing, phosphate-buffered saline formulation in a glass container (Freytag and Templeton, 1998). Ascorbate was added to reduce the methemoglobin content of the dosage form (Vestling, 1942; Gibson, 1943). Optro units contained deoxyHb and were stable at 4–8°C for more than 12 months (Freytag and Templeton, 1998). Subsequently, the composition of the injectable dosage form was changed, first to a 10-g/dl solution of rHb1.1 in phosphate buffered saline, stored at −80°C, and later to formulations comprising 10 g/dl rHb2.0 in a gluconated electrolyte solution, which were stored refrigerated.

Manufacture

Production of totally recombinant hemoglobin presents a number of challenges, each of which was successfully overcome by the rHb team at Somatogen (which was later purchased by Baxter Healthcare Corporation and renamed the Baxter Hemoglobin Therapeutics Division). First, correct assembly of two non-identical α and β subunits as well as correct incorporation of a reduced hematoporphyrin prosthetic group into each subunit is required to obtain a functional hemoglobin (Hoffman *et al.*, 1990). Existing methods for the production and engineering of human Hb in *E. coli* had limited potential as scaleable approaches for commercial production (Nagai *et al.*, 1985). Dual breakthroughs at Somatogen accelerated development. First, the rHb team recognized that in both oxy- and deoxyhemoglobin, the N-terminal residue of one α-subunit and the C-terminal residue of the other α-subunit are only between 2 and 6 Å apart – a distance that can be spanned by one or two amino acids (Shaanan, 1983; Fermi *et al.*, 1984). Crosslinking could be achieved, therefore, by developing structurally conservative genetic fusions of the α-subunit that provide a pseudo-tetramer that cannot dissociate into smaller subunits. To this end, an *E. coli* expression vector was constructed that contained two copies of the α-globin gene fused in tandem by a single codon encoding a glycine residue; this created a fusion junction with the sequence Arginine ($141\alpha_1$) GlycineValine($1\alpha_2$). A heterotrimer was produced thereby, containing in each trimer one dialpha globin plus two beta^{Presbyterian} globins. (The latter mutation on the beta subunit served to decrease the oxygen affinity of rHb1.1, in the absence of 2,3-DPG, to a level roughly comparable to that of native hemoglobin in the presence of 2,3-DPG.)

Secondly, the team addressed historical limitations on heterogeneous subunit expression by constructing synthetic operons in which the expression of the globin subunits was co-regulated. It was found, for example, that co-expression of α- and β-globin favorably influences the intracellular distribution of β-globin and prevents the formation of insoluble inclusion bodies, most probably misfolded protein that is susceptible to proteolysis (Weickert and Curry, 1997). In addition, proper folding of the globin chains apparently is enhanced under conditions in which both subunits are expressed concurrently.

Optimizing rHb1.1 expression required an understanding of the proportion of synthesized protein that is consigned to (1) proteolytic degradation, (2) accumulation as non-native protein aggregates, or (3) proper folding and association into soluble protein, as well as the factors contributing to each fate. The accumulation of soluble recombinant hemoglobin served as a

model system for the study of the effects of factors such as gene dosage, inductor concentration, temperature, protein synthesis rates, and protein accumulation rates on protein solubility in *E. coli* (Weickert *et al.*, 1997). It was found, for example, that a medium globin gene dosage resulted in rHb1.1 accumulating to ~ 7 per cent of the soluble cell protein, of which 78 per cent was soluble. In contrast, a high globin gene dosage (pBR-based or pUC-based plasmids with rHb1.1 genes under the control of the *tac* promoter) effected a three-fold or greater increase in total globin to 23–24 per cent of the soluble cell protein, but 70 per cent was insoluble. Interestingly, production of insoluble protein occurred immediately after induction. Reducing the inducer concentration decreased globin synthesis and increased the proportion of soluble rHb1.1 to 93 per cent. Reducing the temperature from 30°C to 26°C had little effect on the total globin protein synthesis, but increased the percentage of soluble globin protein to about 15 per cent of the total cell protein. These and other optimization strategies enabled development of a model that integrated protein synthesis, folding and heme association (Weickert *et al.*, 1996). Development was capped with a demonstration of high-fidelity translation of recombinant hemoglobin (Weickert and Apostol, 1998).

Although *E. coli* provides recombinant hemoglobin that is capable of incorporating heme provided by the host cell, the process development team found that endogenous heme biosynthesis was not sufficient to supply the high demand for heme in response to strong induction of the globin genes (Verderber *et al.*, 1997). Thus, determinations of turnover of recombinant human hemoglobin and its independent subunits in *E. coli* in the presence and absence of heme demonstrated that the half-life was strongly dependent both on the presence of the pairing subunit and on the presence of heme. Weickert and Curry (1997) concluded that soluble rHb1.1 persistence in the *E. coli* cytoplasm during long periods of stationary phase growth indicated that once assembled, the molecule is extremely resistant to proteolysis.

Development and validation of purification processes that would provide injectable quality rHb1.1 (and subsequent rHbx.x units for clinical trials) constitutes another major achievement of the rHb team. At the bench scale, purification was accomplished by size-selective chromatography on dextran sulfate/Sephadex, followed by strong anion exchange (Mono Q) and strong cation exchange chromatographic separations (Looker *et al.*, 1994). As processing increased in volume and production became more standardized, the purification steps changed to incorporate more time- and cost-effective separations of contaminating *E. coli* proteins and protoporphyrin IX-hemoglobin, bacterial endotoxin and metals. In addition, the 'next-generation' rHbs were subjected to size-selective filtrations to provide hemoglobins having selected molecular sizes (molecular weights).

A general scheme for the manufacture of rHb1.1 and Optro, the trade name for the proposed commercial product containing this recombinant hemoglobin, is provided in Figure 34.2.

For commercial production (Plomer *et al.*, 1998; Looker *et al.*, 2003), seed stock of *E. coli* was thawed and cultured in yeast extract-containing growth media for 10 hours at 37°C to provide sufficient inoculum for seed fermentors. The stock was aseptically transferred to 20-l seed fermentors containing defined growth media and cultured for about 12 hours. The seed fermentor inoculum was then aseptically transferred to a 600- or 1000-L production fermentor containing fresh growth media supplemented with 40 μg/m hemin (bovine hemin was used; Looker *et al.*, 1994). Globin expression was induced by addition of

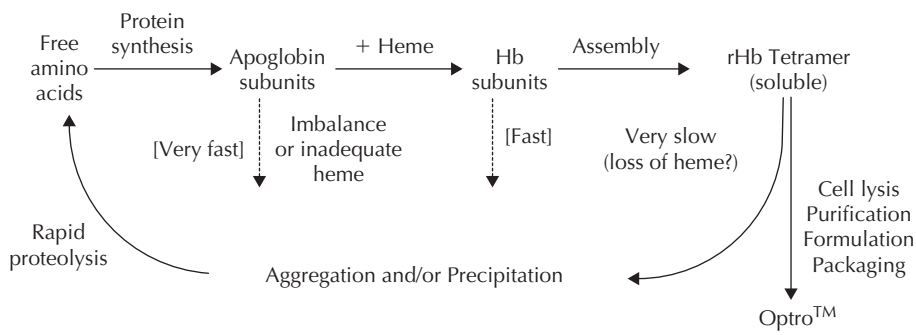

Figure 34.2 Working model for accumulation of stable, assembled rHb (Weickert and Curry, 1997) and overview of the Optro manufacturing process.

β-*D*-thiogalactopyranoside, and glucose was added to the broth in a concentration sufficient to support optimal growth. Cell growth cycles were monitored as optical density, and when sampling indicated that the targeted hemoglobin production and protoporphyrin IX content had been achieved, cells were harvested. Unwashed *E. coli* cells were broken using a homogenizer, and the crude lysates were sparged with carbon monoxide to fully ligate the hemoglobins and then heated at 75–85°C for a length of time sufficient to precipitate protoporphyrin IX-containing hemoglobin but not rHb1.1. The preparation was cooled to ambient temperature, diatomaceous earth was added to facilitate clarification, and the crude lysate was separated from cellular debris and other insoluble materials by filtration. The filtrate, which contained rHb and other soluble proteins, was cooled to refrigerated temperature and treated with zinc acetate to separate crude rHb1.1 from zinc-binding contaminating proteins. The lysate solution was then clarified by depth filtration and applied to an immobilized metal affinity chromatography column charged with zinc acetate solution. The column was washed with Tris buffer, and captured hemoglobin was eluted with 15 mM EDTA, pH 8.5. The composition of the purified hemoglobin solution was adjusted by dilution and the addition of zinc acetate, and then the solution was applied to an IMAC column charged with zinc acetate. After a series of column washes, including washes with 10-mM imidazole solution to remove any remaining *E. coli* proteins, carboxyhemoglobin was eluted from the column with phosphate buffered saline solution. The partially purified hemoglobin solution was ultrafiltered to concentrate the hemoglobin and to exchange the buffer to Tris buffer, pH 8.9. The rHb concentrate was applied to a Q-type strong anion-exchanger resin column to effect a finer purification. The composition of the chromatographic stationary phase(s) changed over time and with scale-up, as improved and/or more appropriate chromatographic media were introduced. Elution with a Tris buffer gradient provided fractions containing purified rHb, which were collected and re-oxygenated. Contaminating metals, particularly nickel, were removed by addition of 1 mM EDTA, incubation and then ultrafiltration. The concentrate was diafiltered into the formulation buffer and the Hb concentration and solution pH were adjusted to the desired values. The formulation was aseptically filled into glass vials that were closed, sealed and stored frozen.

Physicochemical characteristics

In brief, rHb1.1 comprises a pseudo-tetrameric hemoglobin (molecular weight 64.5 kDa) in which the α-subunits are genetically fused by incorporation of a Glycine bridge (i.e., a fusion sequence of Arginine(141α_1)GlycineValine(1α_2)) and the composition of each β-subunit is altered through deliberate substitution of Lysine for Asparagine at position 108 (the Presbyterian mutation). X-ray crystallographic analysis of deoxy rHb1.1 provides coordinate sets that overlay the deoxy native human hemoglobin structure, all in the classic *T* state (Brucker, 2000). The data indicate that neither the genetic fusions that hold the hemoglobin tetramer together nor the valine-to-methionine substitutions at the N-termini of the globin subunits perturb the overall secondary, tertiary or quaternary structure of the protein (Kavanaugh *et al.*, 1992; Kroeger and Kundrot, 1997). The UV-visible absorption spectra of rHb1.1 and native human hemoglobin are superimposable. rHb1.1 displays cooperative oxygen binding, with an oxygen affinity *P*50 at 37°C of about 33 mmHg, even in absence of an effector, and a Hill coefficient nearly equal to that of Hb A_0. Likewise, the NO scavenging rate of rHb1.1 is similar to that of native human hemoglobin. A 10-g/dl solution of rHb1.1 had a viscosity of ~1.9 cPs and colloid oncotic pressure of ~ 40 mmHg.

The second-generation recombinant human hemoglobin, rHb2.0, comprises a pseudo-tetrameric hemoglobin, expressed in *E. coli* and extensively purified, in which the α-subunits are genetically fused and the distal heme pocket of each globin subunit has been genetically manipulated to reduce the rate of NO scavenging roughly 30-fold (Doherty *et al.*, 1998). To extend the circulating half-life following infusion, the protein was derivatized and polymerized using maleimide-capped PEG (Looker *et al.*, 2003). rHb2.0 displays an oxygen affinity *P*50 at 37°C of about 34 mmHg and a reduced cooperativity, similar to the lower cooperativity observed for other polymerized or conjugatively modified hemoglobins. When formulated at 10 g/dl in a gluconated electrolyte solution, the rHb2.0 solution had a viscosity of ~ 2.3 cPs and a COP of ~ 62 mmHg.

Product history

Because the physicochemical properties of the crosslinked hemoglobins DCLHb and rHb1.1 are

nominally quite similar, it is tempting to conjoin the physiological responses to the two hemoglobins as well. There were, however, subtle differences in the magnitude of side effects following their intravenous administration to healthy volunteer subjects, and these differences likely reflect equally subtle differences in the molecular structures of the two types of crosslinked hemoglobin in solution. In particular, the gastrointestinal disturbances that were observed following infusion of rHb1.1 appeared to be more severe than those noted after infusion of other HBOCs (Viele *et al*., 1997; Freytag *et al*., 1998), and a series of studies ensued to identify the causes of the disturbances and, if possible, ways to eliminate them.

In undertaking these studies, the rHb team had two key advantages: (1) the flexibility and agility to design and systematically express multiple series of natural and novel mutants (Weickert *et al*., 1996, 1997; Weickert and Curry, 1997; Weickert and Apostol, 1998), and (2) a rapid mass spectrometric technique for assessing the relative thermodynamic stabilities of the resulting hemoglobins and their constituent components (Apostol, 1999). Briefly stated, through development and optimization of the rHb1.1 process, the rHb team had amassed libraries of data and experience that enabled facile expression of mutant hemoglobins that systematically probed the effects of structural modifications on the physicochemical properties of the resulting hemoglobins. Specific amino acid substitutions incorporated into recombinant hemoglobin modeling naturally occurring mutants or rationally designed hemoglobin variants could be used to modulate its oxygen affinity. Other specific mutations could be used to stabilize hemoglobin to dissociation and oxidation. With the mutants in hand, ion-trap mass spectrometry could be used to differentiate the stability of each hemoglobin, since the amount of applied collision-induced dissociation (CID) energy necessary to break up the intact tetramer into its constituent globin subunits correlates with the stability of the intact tetramer and the heme-containing subunits.

Concurrent with HBOC development, a growing body of studies identified nitric oxide (NO) as a key mediator in the control of important physiological processes, including neurotransmission, inflammation, platelet aggregation and regulation of gastrointestinal and vascular smooth muscle tone (Guslandi, 1994; Beckman and Koppenol, 1996; Walford and Loscalzo, 2003). When findings from these studies were coupled with existing knowledge about NO binding by deoxyhemoglobin, a nexus was established that became a cornerstone for mechanistic rationale for the hypertension responses to hemoglobin preparations (Gould and Moss, 1996; Chang, 1997). With time, both *in vitro* and *in vivo* studies suggested that this rationale could be extended to account for other effects of hemoglobin infusion as well, including for example, gastrointestinal disturbances (Conklin *et al*., 1995; Rattan *et al*., 1995; Hartman *et al*., 1998; Uc *et al*., 1999; Konomi *et al*., 2001), pancreatic dysfunction and biliary dysmotility (Cullen *et al*., 1996; O'Hara *et al*., 1998), and changes in microvascular permeability (Baldwin *et al*., 1998; Baldwin, 1999; Mundy and Dorrington, 2000).

From a broader perspective, however, it was clear that several mechanisms for the hypertension responses to hemoglobin preparations merited consideration, including scavenging of NO (Katsuyama *et al*., 1994; Thompson *et al*., 1994; Sharma *et al*., 1995; Eich *et al*., 1996); increased endothelin synthesis, release, or duration of circulation (Schultz *et al*., 1993; Gulati *et al*., 1995); sensitization of alpha-adrenergic receptors (Gulati and Rebello, 1994; Sharma and Gulati, 1995); and precapillary autoregulation (Vandegriff and Winslow, 1996). Moreover, a rigorous analysis of the multiple pathways for scavenging of NO by hemoglobin indicated that their relative importance had not been adequately deconvoluted.

To extricate the data that were required to gauge the importance of the various mechanisms of action, studies were designed in which the reactivity of hemoglobin toward NO would be altered independent of confounding factors such as differences in molecular volumes or colloid osmotic pressures. To this end, a set of recombinant hemoglobins that varied in rates of reaction with NO were synthesized (Doherty *et al*., 1998). In an initial round of mutagenesis, substituted alpha subunits were paired with wild-type beta subunits and *vice versa*. The reaction of NO with oxyhemoglobin forms of such constructs often yielded biphasic time courses due to different reactivities of the mutant and wild-type subunits. Fitting these time courses to a two-exponential function yielded the reaction rates of both subunits. By repeating this process for a larger number of mutant constructs, sets of α and β subunits having a wide range of rate constants for direct NO oxidation ($k'_{NO,ox}$) were obtained. The data showed that the rate constant of the oxidative reaction is, in fact, slightly greater than that for NO binding to deoxyhemoglobin, implying that

the NO-related physiological effects caused by acellular Hb reflect NO scavenging by both oxyHb and deoxyHb. Equally importantly, the analysis gave authoritative perspective to the relative insignificance of interactions of NO with acellular methemoglobin or reactions involving intravascular S-nitroso Hb.

Replete with new recombinant hemoglobins having significantly reduced reactivity to NO (Doherty *et al.*, 1998), development of a second-generation rHb was initiated. (The genes encoding the tandem fusion of the α-subunit were retained in the new mutants.) One such new rHb, rHb3011, in which site-directed mutagenesis was effected at the distal heme pocket, exhibited a nitric oxide reaction rate constantly 30-fold slower than rHb1.1 and properties, such as oxygen affinity and circulating half-life in rodents, that closely matched those of rHb1.1 (Eich *et al.*, 1996). An investigation that compared the gastric motility effects of rHb1.1 and rHb3011 in rats illustrates some of the differences in NO-reactivity and physiological effects between the two hemoglobins (Eich *et al.*, 1996; Hartman *et al.*, 1998). (In this study, increasing doses of L-NAME (1, 3, and 10 mg/kg, intravenously) were used as a positive control to demonstrate that this rat model of gastric dysmotility is sensitive to reductions in endogenous NO.) Each variant was formulated at 10 per cent Hb in formulation buffer (150 mM NaCl, 5 mM sodium phosphate, 4 μm EDTA at pH 7.2), and then administered intravenously to fasted rats in doses of 750 and 1500 mg/kg prior to administration by gavage of 3 g (approximately 3 ml) of a defined meal. The percentage of meal emptied was determined 45 minutes after feeding. rHb1 reduced gastric emptying significantly (48 per cent and 71 per cent, respectively, relative to human serum albumin controls), whereas the effect of rHb3011 was significant only at the higher dose (42 per cent, $P < 0.05$).

Although the hypertensive effects of HBOCs in the pulmonary circulation are not as well documented as those in the systemic circulation, several reports have described pulmonary vasoconstrictor/contractile responses secondary to exposure to hemoglobin (Freas *et al.*, 1995; Heller *et al.*, 1998). A second comparator study was completed to determine whether HBOCs with reduced NO scavenging capacities might be advantageous in minimizing the potential for development of pulmonary hypertension and subsequent edema formation (Resta *et al.*, 2002). In this study, the effects of rHb1.1 and rHb2.0 on vasoconstrictor reactivity and vascular permeability in isolated, saline-perfused rat lungs were examined. rHb1.1 enhanced pulmonary vasoconstrictor reactivity to both hypoxia and the thromboxane mimetic U-46619 (9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin F_{2a}, Cayman Chemical Co.) in dose-dependent fashion. In contrast, rHb2.0 produced little or no change in reactivity to these stimuli. These findings are consistent with an effect of endogenous NO to attenuate both hypoxic and U-46619-induced pulmonary vasoconstriction. It was further demonstrated that, whereas rHb1.1 abrogated pulmonary vasodilation to the NO-donor S-nitroso-N-acetylpenicillamine (SNAP), dose-dependent responses to SNAP were preserved (albeit attenuated) in lungs treated with rHb2.0. Despite markedly enhanced reactivity to vasoconstrictor stimuli in isolated lungs treated with rHb1.1, no apparent effect of either hemoglobin solution on baseline vascular resistance was observed. The capillary filtration coefficient was unaltered by either rHb1.1 or rHb2.0. Likewise, no differences in lung wet-to-dry weight ratio were observed, other than differences consistent with a greater vascular hydrostatic pressure caused by a 20-mg/ml dose of rHb1.1. Neither Hb measurably altered microvascular permeability.

These studies, as well as others not yet published, encouraged the initiation of clinical trials in which the safety of rHb2.0 was assessed following intravenous administration to healthy volunteer subjects. The disheartening findings culminated in a negative program review that, when coupled with a major shift in corporate strategy by Baxter, ended development. The program was discontinued in 2003.

RETROSPECTIVE

This chapter may well document the end of the road for clinical applications of hemoglobin tetramers similar to DCLHb or rHb1.1. It is important, therefore, to capture a few of the significant gains associated with their development.

Although a few broad postulates provided general blood substitute product objectives when DCLHb and rHb development were initiated, little guidance was available concerning specific product and formulation questions. For example, only speculative guidelines suggested what hemoglobin concentration, oxygen affinity or formulation would be most appropriate. Little reliable

guidance was available concerning colloid oncotic pressure effects of the hemoglobin, nor was there authoritative information on the optimal circulating half-life, volume of distribution, point at which intravascular methemoglobin content exceeded tolerable values, hemoglobin metabolism, and so forth. Through the progress that they reported, both the DCLHb and the rHb teams educated the HBOC community as well as broader networks of scientific and clinical investigators.

When development programs were initiated, no standard protocols were available for *in vitro* or *in vivo* testing of the various HBOC preparations. Indeed, differences in experimental protocols (top-load administration to normovolemic animals, exchange transfusion, resuscitation from hemorrhage, resuscitation from hemorrhage of dehydrated animals, etc.) continue to complicate interpretation of published data and standardization of test regimens for potential new HBOCs.

Two advances stand out from among the many associated with DCLHb development. First, the DCLHb development team raised the bar and established new quality standards for hemoglobin processing. An integrated approach for converting Hb to an HBOC was developed that addressed manufacturing challenges such as the exclusion of pathogenic contaminants, a maintenance of the lack of antigenicity and immunogenicity exhibited by native human hemoglobin, and the ability to provide an active oxygen carrier with minimal methemoglobin content. The confidence that DCLHb would exhibit the same properties and performance characteristics, batch after batch, month after month, year after year, encouraged investigations of its therapeutic benefit.

Secondly, the DCLHb literature is remarkable for the diversity of applications that were studied and reported. While the initial focus was on traditional applications as a 'blood substitute', the DCLHb literature was the first to acknowledge and espouse the fact that acellular hemoglobin is a pharmacologically active biologic that has both oxygen-delivery action as well as physiological actions unrelated to delivery of oxygen. This body of work also showed that it was reasonable to anticipate that an acellular hemoglobin will exhibit dose-dependent but self-limiting vasoactivity with a duration of hypertension that depends on the concentration of intravascular oxyhemoglobin. Likewise, these reports showed that after infusion of DCLHb, the protein remained intact and underwent a slow oxidation to methemoglobin as it circulated systemically (Bush *et al.*, 1994). Further, the 'pseudo-jaundice' that was frequently noted in patient summaries verified DCLHb metabolism similar to that of native hemoglobin. Finally, the term 'Bridge to [red cell] transfusion,' which was introduced by the DCLHb team, has since been applied to every other HBOC in clinical trials.

Likewise, the rHb team merits accolades for the breadth and depth of their 'structure–activity relationship' studies on a biologic. In particular, the application of mass spectrometric tools to determinations of rHb stoichiometry and stability afforded a sizeable bibliography of data concerning the effects of amino acid substitutions in the globin subunits and within and outside the region of the heme pockets. These data, when combined with data concerning the effects of the substitutions on parameters such as expression in *E. coli*, oxygen affinity, NO oxidation, and stability toward oxidation, denaturation, and aggregation in solution, afford powerful guidance for the genetic engineering of hemoglobin.

Another significant outcome of the extensive studies required for rHb development was a series of reports which alerted investigators around the world to potential problems associated with Hb production and, more generally, with production of certain peptides. For example, investigators had long assumed that methemoglobin content could be decreased or maintained at low levels by treatment with reducing agents such as thiols, dithionite or ascorbate without affecting the hemoglobin molecule. Kerwin *et al*. (1999) showed that reduction of methemoglobin with ascorbate may give rise to ascorbate-modified hemoglobins and/or induce aggregation and precipitation and provided insights into the mechanisms of these undesirable changes. Similarly, Levine *et al*. (1998, 1999) reported studies of oxidative deamination and intramolecular crosslinking that occurred when nickel catalyzed the oxidation of X-Histidine N-terminal peptides. Since nickel is frequently employed in immobilized metal affinity chromatographic separations, an understanding of the mechanism of this unexpected reactivity is key to its prevention. Likewise, Lippincott and Apostol (1999) identified potential artifacts of peptide mapping that could be introduced during digestions in the presence of urea, and presented approaches to avoid the carbamylation of cysteine.

In summary, we have witnessed meteoric progress in our understanding of HBOCs as potential clinical alternatives to red blood cell

transfusions. Original predictions about their clinical utility have been circumscribed. The anticipation that the solutions would be viable oxygen transport vehicles for all clinical situations where red cells are transfused has diminished to the reality that at best, for certain acutely anemic or hypovolemic patients, the solutions may be bridges to transfusion of red cells. The hope that the oxygen carriers would be useful to correct local oxygen deficits where red cells are not indicated (PTCA and stroke, for example) have been dashed. Baxter's unusual policy of engaging the broader scientific community in study of DCLHb and rapid publication of the results has not been emulated by other companies, and critical information is not yet available about issues such as focal physiological effects of circulating hemoglobin, hemoglobin metabolism and excretion, and adverse effects related to oxidation of lipids, proteins, and cells by circulating hemoglobin. The possibility that these HBOC solutions present significantly enhanced safety relative to that of stored red cells remains an open question.

EDITOR'S SUMMARY

Cell-free hemoglobins with molecular weight 64 kDa have been extensively studied. There are two types, one chemically crosslinked and the other produced in a recombinant (*E. coli*) system. In the first, the α chains of hemoglobin were covalently crosslinked between Lysine 99 residues with the reagent DBBF (bis(dibromosalicyl)fumarate). The product was called $\alpha\alpha$-Hb by the US Army and DCLHb by Baxter, who produced the same product, initially under contract to the Army. The second was rHb1.1, produced by Somatogen. This recombinant molecule was also engineered to prevent dissociation of tetramers to dimers.

At the outset of development of these products, there was the widespread assumption that crosslinked hemoglobin with many properties (such as *P*50) that mimic red blood cells would be ultimately successful as a blood replacement. Before this could be proven conclusively, and because of the great competition and pressure to commercialize a product, the methods for production were developed before the scientific foundation for clinical implementation was laid. Consequently, clinical trials were disappointing, mainly due to vasoconstriction and its consequences. Both products were eventually abandoned.

Although this is a disappointing story in one way, in another it was a great success: a very large body of scientific literature was generated which helped define the remaining problems in the field. Research on subsequent products could therefore take advantage of this extensive body of work. Products such as polymerized hemoglobin and conjugated hemoglobin (particularly to PEG) may have overcome some of the limitations of the 64-kDa class of products, although at this time they have not been studied as intensely in clinical trials.

Conflict of Interest statement

Deanna J. Nelson is President and Chief Scientific Officer of BioLink Life Sciences, Inc., a life sciences technology innovator company that is developing novel pro-drugs and drug analogs. Dr Nelson is not an employee or board member of a company that is developing a 'blood substitute'.

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Clinical Studies with DCLHb

Chapter 35

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INTRODUCTION

The clinical development pathway mapped out for DCLHb in the late 1980s was simple: show that DCLHb is safe and effective in angioplasty. A perfluorocarbon product had recently been approved for that indication, and the preclinical studies in several animal models of coronary angioplasty showed that DCLHb was more effective than the perfluorocarbon in preventing ischemia and ventricular tachycardia during balloon inflation (Vincent *et al.*, 1991, McKenzie *et al.*, 1994). Potential clinical investigators in Salt Lake City and San Diego were eager to start human studies in that indication. Their experience with the perfluorocarbon was that although effective, it was difficult to use. Since DCLHb was packaged in the oxygenated state, it could be infused directly from the bag into the coronary arteries. This posed a tremendous logistic advantage over the perfluorocarbon, which had to be mixed and oxygenated.

The Phase I study, as discussed with FDA physicians in October 1990, was to be a crossover, increasing dose study of 250, 500, 750 and 1000 ml of the 10 per cent DCLHb solution. The design of the study was lauded by the FDA as the first truly scientific Phase I study to be done with a hemoglobin solution, in contrast to the usual 'throw it in and see what happens' approach to that point. Potential study sites in Lincoln (Nebraska) and Belfast (Northern Ireland) were being prepared, the latter in case the Investigational New Drug (IND) approval was delayed. It was anticipated that the clinical study would begin in spring 1991.

PRECLINICAL CARDIAC LESIONS

All was going well until results of a primate toxicity study disclosed the presence of heart lesions (Burhop *et al.*, 2004). Other animal species at that point had not expressed this toxicity. The dose-ranging study in rhesus monkeys showed a 'no effects dose' up 200 mg per kilogram. This correlated to less than 200 ml of the 10 per cent DCLHb solution in an 80-kg individual, which was below the anticipated volume needed for angioplasty. Thus, Baxter placed the clinical studies plans on indefinite hold.

The lesions observed in the rhesus monkey hearts were subsequently reproduced in swine. They were described as microscopic areas of necrosis occurring in <3 per cent of the heart muscle, predominantly in the left ventricle, and observed most clearly within 1 week of dosing. Often the lesion was no longer visible by 2 weeks, which was thought to explain why it had not previously been identified with either DCLHb or other hemoglobin solutions.

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The nature of the lesion was virtually identical to that described approximately 25 years earlier in human hearts after prolonged exposure to catecholamines at clinical doses (Haft, 1974). Platelet aggregation with resulting micro-ischemia was hypothesized to be the cause of the lesions observed in human hearts at autopsy. Samples of the rhesus and swine heart tissues were brought to the pathologist first describing the lesion in humans; he confirmed that the hemoglobin-induced and catecholamine-induced lesions were virtually identical.

More than a year of preclinical studies ensued, attempting to understand and prevent these lesions in the animal models. Anti-inflammatory agents, anti-histamines and prostaglandin blockers were tested, but none prevented or reduced the lesions. Since DCLHb was shown to increase blood pressure, it was hypothesized that a vasoconstrictive effect might be responsible for the lesions. Studies with various antihypertensive agents in which blood pressure increase was effectively blocked did not show an absence or reduction in heart lesions, however.

PHASE I STUDY

The heart lesion studies results were brought to the FDA along with a revised clinical development proposal in early 1992. The Phase I study, still of crossover design and involving normal healthy volunteers, would begin at approximately 1/10th the dose previously proposed: 25 mg/kg. Dose increasing from 25 to 50, 100, 200 and 300 mg/kg was suggested, with close monitoring of myocardial enzymes and function for evidence of injury. An IND application was submitted, then approved in the minimum time, permitting the Phase I study to begin at the Lincoln facility in July 1992.

Eight volunteers per dose group were to be studied in a crossover design, each randomized to receive either DCLHb or lactated Ringer's solution as an initial infusion, and then the other solution 7 days later (Przybelski *et al.*, 1996). The volunteers remained in the study center for 16 days total. Infusions were done in a double-blinded manner with two discrete clinical teams involved in the study.

A pressor effect of DCLHb was apparent with the lowest dose. The 50- and 100-mg/kg dosing groups showed the same effect, with slightly greater increase of pressure in the 100-mg/kg dose group. The diastolic blood pressure was primarily affected, with an average increase of 10–15 mmHg. A corresponding bradycardia was also observed, with no clinical symptoms associated with the approximately 5 beat per second slowing of the heart rate. As in the preclinical studies, the mechanism of the pressor effect was not understood.

The one serious adverse event in the study was a short run of ventricular tachycardia that occurred in the control infusion phase in one young, healthy individual. This pre-empted the subsequent crossover to DCLHb for that subject.

The most concerning adverse event in the DCLHb recipients was the frequent occurrence of epigastric discomfort. This was typically experienced within 4 hours of dosing, and resolved without treatment or sequelae. Appetite was not affected, and in most cases the subject felt better after eating. There was no accompanying vomiting, which was significant since some canine and a few primates in the preclinical studies had responded to DCLHb infusion with such a reaction (unpublished data). In the animal studies the cause of the gastric effects was never discerned, but in the Phase I study gastric reflux or esophageal spasm seemed the most likely causes based on clinical presentation.

PHASE II STUDIES

Hemorrhagic shock study: first entry into patients

With a pharmacologic effect clearly demonstrated with the first three doses of DCLHb in the Phase I study, Baxter proposed that rather than continue the dose escalation and normal healthy volunteers, the product be moved to a clinical setting where the pressor effect could potentially have some clinical benefit. Preclinical studies in rats and swine hemorrhage models provided evidence that the pressor effect actually increased major organ perfusion, rather than causing the ischemia that would result from a generalized vasocontrictive effect (Przybelski *et al.*, 1991; Sharma and Gulati, 1994). The rat and swine pressor data, which could be superimposed on the human blood pressure response, predicted that the pressor effect was limited, and that a maximum pressure increase was observed at approximately 100 mg/kg, with virtually no increase with higher doses (unpublished data). The animal data

also showed that any of the commonly used antihypertensive agents could mitigate the pressor effect. Thus, after a Phase I review meeting, the FDA permitted Baxter to transition to patient studies rather than continue dose escalation in normal volunteers.

While the Phase I study was ongoing, a total of 11 sites had been prepared in the US and Europe for a hospital-based study of hemorrhagic shock. The FDA agreed to changing to a volume-based increasing dose approach, such that 50, 100 and 200 ml of DCLHb were infused into patients experiencing American College of Surgeons class III or IV hemorrhagic shock, in an increasing dose manner (class I, blood volume loss up to 15 per cent; pulse $<$ 100, blood pressure normal; class II, 15–30 per cent blood volume loss; pulse $>$ 100, blood pressure normal; class III, 30–40 per cent blood volume loss; pulse $>$ 120, blood pressure decreased; class IV, $>$ 40 per cent blood volume loss; pulse $>$ 140, blood pressure decreased; see American College of Surgeons, 1997).

The study commenced in March 1993, with 139 patients enrolled in it. Fewer deaths occurred in the DCLHb recipients than in the saline control group (13 vs 16), and there were significantly fewer cardiac rhythm disturbances and cardiac events in the DCLHb group. The same pressor effect was observed in hemorrhage patients as in the Phase I study normal volunteers (Przybelski *et al.*, 1999).

The major drawback in the study was the difficulty in enrolling patients at those levels of shock. This resulted in a protocol amendment changing the inclusion criteria to include the less severe Class II hemorrhagic shock. Despite this relaxation of criteria and a large number of sites for a Phase II study, it still took almost 2 years to complete.

Low-dose surgery studies

In an effort to further study low doses of DCLHb for both potential cardiac toxicity and possible clinical benefit of the pressor effect, intraoperative studies were started in Europe in parallel with the hemorrhagic shock study. Production of DCLHb had been moved from California to Belgium for scale-up in a Baxter facility; this facilitated larger batches for multiple clinical studies. The move also supported an accelerated clinical studies plan. Since the product was no longer made in the US, FDA approval of studies was only necessary when US sites were involved. Thus, multiple studies could be run in parallel to rapidly advance dosing and develop the safety profile and potential clinical applications for DCLHb.

The first surgical studies infused DCLHb into orthopedic surgery patients at the time anesthesia was induced. The first study investigated the same doses as tested in the Phase I study, along with a 200-mg/kg dose, also in an increasing dose regimen (Remy *et al.*, 1999). Blood utilization and hypotensive episodes were the efficacy endpoints evaluated in this double-blinded study. In total, 80 patients were rolled at two sites in Belgium. While there appeared to be a significant reduction in blood utilization after the first dose, this did not hold true for the entire study. There were fewer perioperative hypotensive episodes in the DCLHb-treated group. No serious adverse events and no consistent adverse events attributable to DCLHb occurred, so further surgical studies in more complicated patients ensued.

A multicenter aortic aneurysm repair study was initiated with similar safety and efficacy endpoints as the orthopedic study (Garrioch *et al.*, 1999). In that randomized, controlled trial, 50, 100 or 200 mg/kg of DCLHb was infused prior to general anesthesia. DCLHb was shown to be vasoactive, producing an increase in blood pressure as high as 25 per cent above baseline across the three dose groups, with a slight decrease in cardiac index and calculated oxygen delivery. The pressure increase persisted for several hours beyond the infusion period, providing a degree of cardiovascular stability during the operative procedure. Two cardiac events and three cases of renal failure occurred in the control group, with none occurring in the 34 DCLHb-treated patients.

Hemodialysis

The Baxter Renal Division was aware of the pressor properties of DCLHb, and through their hemodialysis contacts at Hennepin County Medical Center a proposal was made to to use DCLHb to prevent the hypotension frequently observed during that procedure. It was pointed out in the study proposal that hypotensive episodes often limit the duration of dialysis, resulting in suboptimal treatment, hospitalization and, rarely, cardiac events, stroke and death.

Eighteen dialysis patients were treated with 25, 50 or 100 mg/kg of DCLHb or saline in a crossover design study (Swan *et al.*, 1995). As predicted, the hemodialysis patients had fewer hypotensive episodes when pre-treated with

DCLHb. The safety profile was also favorable in these closely monitored, chronically ill patients.

The results were brought to the FDA for discussion of a possible clinical indication in hemodialysis, with the efficacy endpoint being a composite of the clinical organ dysfunction resulting from hypotension. Twelve large hemodialysis centers had organized to do a Phase II/III study, and Baxter produced enough product in 50-ml bags to complete the study. The ambivalent response by the FDA to this unorthodox use of a hemoglobin product, however, coupled with Baxter's stated objective of developing a 'blood substitute', resulted in shelving what might have been the first approved hemoglobin clinical application.

Intensive care unit studies

Four patient studies in various clinical settings established a 'no effects dose' (200 mg/kg) with no evidence of myocardial insult; there were no enzyme elevations or cardiac rhythm disturbances attributable to DCLHb use. Furthermore, the hemorrhagic shock and abdominal aortic repair studies provided some evidence of cardiac protection. However, the 200-ml dose used in those studies was far less than the 500 ml or more thought to be necessary to use DCLHb as a blood substitute.

In an effort to establish dosage guidelines in septic shock and systemic inflammatory response (SIRS) patients, an ICU study was conducted in the UK involving patients who failed to respond to standard pressor therapy with catecholamines and vasopressin (Rhea *et al.*, 1997). These decompensating patients were infused with 100, 200, 300, 400 or 500 ml of DCLHb in 100-ml increments, given hourly as needed to support pressure and perfusion.

Fourteen patients were enrolled over approximately 1 year, and the pressor effect seemingly benefited some of them. The potent vasoactive effect was observed within 5 minutes of starting infusion, and persisted for up to 72 hours. The main efficacy endpoint, reduction of concomitant vasopressor treatments, was attained. Four of the patients unexpectedly survived the severe shock, and infusion of DCLHb did not appear to contribute to the demise of the non-survivors.

The success in the shock study encouraged an investigator (J. Lipman) from the Bagwaneth Hospital in Johannesburg, South Africa, to pursue the use of DCLHb in the ICU setting. He proposed to gather oxygenation data by measurement of oxygen consumption in anemic, septic shock patients by analysis of expired gases. Patients were randomized to receive 250 ml of DCLHb, 500 ml of DCLHb, or packed red blood cells, when a transfusion was indicated for anemia. The study took almost 2 years to enroll 19 patients, but provided the best oxygenation data produced in the DCLHb clinical studies: oxygen consumption increased in the DCLHb patients even though oxygen transport was less than in the blood recipients, suggesting that cellular oxygen utilization was enhanced by treatment with the hemoglobin solution.

PHASE III STUDIES

Cardiac surgery: the pivotal efficacy study

Several investigators involved in the early surgery studies proposed a blood-sparing efficacy study of DCLHb in cardiac surgery. Anesthesiologists and cardiac surgeons from eight sites in Europe designed a study in which 250 ml of DCLHb would be infused when the clinician decided that a blood transfusion of the first unit of packed red blood cells was indicated. The patients were post-bypass pump, coronary artery grafting and/or valve replacement recipients who required perioperative transfusion. The fact that they required transfusion would make them a relatively high-risk subset of the usual cardiac surgery patient. Based on the safety data through 500 ml of DCLHb, it was decided that up to three 'units', a total of 750 ml of DCLHb, would be infused within 24 hours post-bypass.

After a day of deliberation, a set of transfusion triggers was identified by the investigators as the grounds for transfusion. They also agreed that prior to transfusion, a form would be completed identifying the indication for the transfusion. Another form would be completed after transfusion or infusion of DCLHb, indicating clinical response to the treatment. This was done for each of the three potential transfusions/infusions.

The randomized, single-blinded study enrolled 209 patients in less than 2 years, with 104 patients placed in the DCLHb group (Lamy *et al.*, 1999). Most of those patients received the maximum dose of 750 ml. Nineteen per cent of the patients receiving DCLHb received no packed red blood cells, suggesting that the hemoglobin solution effectively supported these patients in whom a transfusion had been clinically warranted and

documented. Perhaps the most impressive aspect of this result was that the need for transfusion occurred after transfusion-sparing efforts, including cell-saving and pro-coagulation therapies, failed to obviate the need for blood.

The hemodynamic effects of DCLHb included a consistent, slightly greater increase in systemic and pulmonary vascular resistance with associated increases in systemic and pulmonary arterial pressures compared with packed RBC infusion effects. Cardiac output values decreased more in the DCLHb-group patients.

Positive findings for DCLHb in the study included fewer deaths in this high-risk surgical group: eight died in the blood transfusion group compared to six in the DCLHb group. The DCLHb group also had significantly lower troponin I levels at 72 hours post-bypass. There were no significant adverse events attributed to the study product.

On the negative side, the study was stopped temporarily when investigators observed jaundice in many patients receiving a 500 or 750 ml dose of DCLHb. There was no consistent corresponding change in liver enzymes, but bilirubin was elevated in those patients. Extravasation of DCLHb into the skin with subsequent degradation may have been responsible for this observation. Other adverse effects occurring more frequently in the DCLHb group were hypertension, increased serum glutamic oxalo-acetic transaminase, abnormal urine, and hematuria. There was one case of renal failure in each group.

Pancreatitis

A clinical safety study initiated in Germany during the cardiac surgery study raised the specter of a more significant adverse effect – pancreatitis. The third patient receiving DCLHb developed severe abdominal pain and was found to have pancreatitis with a pseudocyst, resulting in an ICU admission and a prolonged hospital stay. While the patient had a potentially contributory past medical history and was on multiple other medications, the temporal association with infusion implicated DCLHb.

An expert panel convened by Baxter and attended by an FDA representative reviewed all abdominal pain adverse events and enzyme results from the various studies. As of September 1997, pancreatitis had been observed in six DCLHb recipients and two control patients across a total of 800 patients (nearly equally divided) in the various studies. Three of the cases were considered by investigators as 'product-related', with two of those classified as serious adverse events. Elevated amylase and lipase values were also observed more frequently in the DCLHb groups.

The complexity of the clinical settings in which the pancreatitis cases occurred, along with the relatively few cases in more than 400 DCLHb recipients, led the panel to conclude that DCLHb was not the obvious cause of pancreatitis. Nonetheless, it stressed that surveillance needed to be increased such that any adverse event of abdominal pain warranted a pancreatitis work-up, including a CT scan of the abdomen. Animal studies to assess the effect of DCLHb on pancreatic function were also recommended. The various models of chemical pancreatitis and pancreatic ischemia failed to replicate the clinical findings (unpublished data). Sphincter of Oddi tone did increase in response to DCLHb infusion in the Australian opossum pancreatitis model, however (Konomi *et al.*, 2000).

EUROPEAN PRODUCT APPLICATION FOR CARDIAC SURGERY

By the time the cardiac study was completed, approximately 400 patients had received DCLHb. Studies which had been started during the cardiac surgery study included an acute ischemic stroke trial and a surgical study in the US. In the latter effort patients experiencing high blood loss received up to 750 ml of DCLHb in a double-blinded study with design similar to the cardiac surgery study.

Thus, DCLHb appeared on track for product approval. Overall, the safety profile appeared quite favorable at the relatively low doses tested. The fewer DCLHb group deaths in the hemorrhagic shock study and the cardiac surgery study results of fewer deaths and lower troponin I levels in the DCLHb patients, coupled with oxygenation results from the South African ICU study, placed this hemoglobin product in a very positive light in early 1997. From a clinical development standpoint, Baxter had gone from having no human data in early 1992 to the completion of a large efficacy study in less than 5 years, making it the acknowledged leader in the field at that point.

Despite the fact that fewer than 100 patients had been treated with the maximum dose of 750 ml used in the cardiac surgery study, Baxter decided

to file for product approval. The rationale was that the ongoing studies in the US and Europe would produce several hundred more patients infused with at least 750 ml of DCLHb by the time the final decision on the product application would be made. These data would be available for member country review if desired. Thus, the central application through the EMEA was initiated, and a formal submission was made to and reviewed by the 12 European Community countries at that time. The clinical expert report accompanying the submission argued that DCLHb could be used safely in cardiac surgery when blood was not available.

The result of the submission was that DCLHb was not approved. The overwhelming safety concern was the low number of the patients studied in the proposed clinical application, cardiac surgery. From an efficacy standpoint, only one of the two primary endpoints had been achieved in the study: a significant percentage of patients had avoided blood transfusion, but the total number of units transfused was not significantly reduced in the DCLHb group. Another reason given for the dismissal was the five-to-one number needed to treat ratio – that is, only one patient avoided transfusion for every five that were exposed to DCLHb. This was considered unacceptable given the safety of the European blood supply compared with the potential toxicity of DCLHb. Again, the concern was based primarily on the small number of patients treated with higher doses of DCLHb to date.

Instead of revamping the cardiac surgery protocol and keeping this as a first product approval indication, no further work was done in this area. The decision had been made to complete the US surgery study and focus on the US and European trauma studies that were being developed. Trauma was the marquee indication at every presentation of the potential use of DCLHb. The master plan was to bolster patient treatment numbers via the trauma and general surgery studies' enrollments, then combine the results into a comprehensive 'blood substitute' submission for both US and Europe when those studies were completed.

TRAUMA STUDIES: THE BEGINNING OF THE END

A major hurdle for the US trauma study was the need to circumvent informed consent, given that the product was to be used in emergency situations where often patients were incapacitated and family members unavailable (Sloan *et al.*, 1999a). The FDA was already involved in drafting guidelines for such studies, so the Baxter trauma study became a test case. The FDA also convened an advisory panel to review the proposed study in a public forum. The panel recommended the study be permitted, although there was concern that the 28-day survival endpoint could not be achieved in such a heterogeneous population of patients, which included blunt as well as penetrating trauma.

After the major effort of community notification for lack of the informed consent, the study laboriously enrolled patients at 18 sites toward a target of over 800 patients. Patients presenting to emergency departments with severe traumatic hemorrhagic shock and unstable vital signs or critical base deficits were randomized to receive up to 1000 ml of DCLHb or saline as per standard care (Sloan *et al.*, 1999b). Prior to the first safety interim analysis, however, there were major concerns regarding the 28-day mortality rate for those treated with DCLHb. Of the 52 patients in the DCLHb group 24 had died, compared to 8 of the 46 control patients. The safety committee recommended cessation of the study (Lewis *et al.*, 2001).

The interim findings were a surprise to investigators: none of them voiced concerns either during the study or at the meeting to review the condemning interim data. It was pointed out that six of seven traumatic cardiac arrest victims had been randomized to the DCLHb group, as well as the majority of penetrating chest trauma patients. A review of the trauma severity scores, however, did not support the possibility of a randomization bias as being responsible for the disproportionate number of deaths in the DCLHb group.

The trauma study in Europe, which was an ambulance-based, 'on scene' study involving approximately 50 sites in multiple countries, enrolled 121 patients before being prematurely terminated (Kerner *et al.*, 2003). There was no increase in mortality at either the 5- or 28-day endpoints in that study. Median volumes of cumulative blood products were less for the DCLHb recipients at 1 and 7 days. Despite a lack of safety issues, Baxter chose to terminate that study as a result of the safety concerns in the US trauma study.

Stroke study

If there was any question as to whether DCLHb should be continued to be developed after the trauma results, the stroke clinical study results put that to rest. Preclinical studies showed a remarkable neuroprotective effect of DCLHb in the rat, rabbit and cat models of neuroischemia, and a rat middle cerebral artery occlusion study suggested that the pressor effect was advantageous for reducing the size of the stroke in that model (Cole *et al.*, 1992). Thus, a four-center European safety study used DCLHb to treat ischemic stroke patients with hemiparesis, with low-volume doses given every 6 hours over a 3-day period to theoretically enhance perfusion of the brain area at risk (Saxena *et al.*, 1999). DCLHb recipients did worse at every dose tested, however, leading the investigators to conclude that the only thing worse than having a large stroke was to also receive DCLHb.

The discrepancy between the preclinical and clinical stroke study results may have rested in the fact the animal studies used a single large-volume top load rather than repeated small doses. In the clinical study regimen, patients would potentially get the pressor effect without the added volume that may have been critical for safety. If not for the disturbing trauma study results, the stroke study might have been viewed as an anomaly, such that DCLHb would have simply been contraindicated for use in stroke patients.

THE END OF DCLHb

The US major blood-loss surgery study was also halted as a result of the trauma safety issues, and a review of the patient results was completed by the principle investigator (Schubert *et al.*, 2003). A similar number of surgical patients was spared blood transfusion through 7 days post-operation when treated with DCLHb (23 per cent), but a less favorable adverse events profile emerged compared to the cardiac surgery study, including cases of pancreatitis. Thus, the distinctly negative results from the US trauma study, the stroke study and, to a lesser degree, the US surgery study prompted discontinuation of the DCLHb clinical program in 1998, after more than a dozen clinical studies in a variety of clinical settings.

LESSONS LEARNED

With the favorable clinical picture prior to the US trauma study, a larger number of DCLHb-treated cardiac surgery patients showing similar mortality and morbidity outcomes might have supported a successful product application for that specific indication; the lesson learned is to keep the indication focused and the numbers of patients treated large enough to support the safety as well as the efficacy of the blood substitute. A more subtle lesson, however, is that avoidance of blood transfusion is not necessarily an acceptable efficacy endpoint, given the regulators' attitudes about the improved safety of the current blood supply. In the case of DCLHb and cardiac surgery, the decreased mortality or lower troponin I levels (a surrogate marker of cardiac ischemia) observed in the DCLHb group could have been advanced as the endpoint(s) for a larger, confirmatory/Phase III study.

The terminal event for DCLHb was the US trauma study. Trauma, as a clinical indication for product approval, is certainly not practical, and may be impossible. The heterologous nature of the patient population, along with the facts that the vast majority of patients will survive their injuries regardless of whether or not they receive any pre-hospital treatment, and that those who die from trauma are usually unsalvageable, makes the mortality endpoint of trauma studies unrealistic.

Thus, if proof of efficacy in trauma is mandated for product approval in any indication, then no blood substitute will be approved for routine clinical use. In view of the US trauma study results, however, and given the likelihood that a blood substitute will be used for trauma patients regardless of the indication it is approved for, it is reasonable for the regulatory authorities to require a demonstration of safety in that patient population. A relatively small, randomized safety study for the product approval submission, followed by a large, open label, post-marketing study for ongoing safety monitoring, would seem an effective and reasonable approach to that end.

EDITOR'S SUMMARY

The product developed by the US Army ($\alpha\alpha$-Hb) and Baxter (DCLHb) is the most intensively and widely characterized and studied hemoglobin-based blood substitute. Baxter developed a cGMP production process that permitted the large-scale clinical evaluation which is summarized in this chapter. In some ways, the development reflects the changing requirements for a blood substitute and perceptions of blood safety over the period of this development.

The finding of microscopic lesions in hearts of susceptible animals cast doubts over the maximal doses that should be used in human trials, although the lesion was never demonstrated in humans. Nevertheless, initial reaction was to focus on low-dose indications such as regional perfusion (PTCA). Phase I trials showed the hypertension that had been found in animal studies, but other side effects were considered harmless.

As development evolved and results seemed positive, larger potential markets than PTCA, such as cardiac surgery and trauma, suggested a wider set of clinical studies. Again, initial Phase II trials did not show side effects that would halt clinical development. Therefore, larger, multicenter Phase III studies were performed both in the US and Europe. These showed conclusively that DCLHb was unsafe as given, and further clinical development was halted.

The case of DCLHb should be carefully studied by any company or any researcher considering entering this field. It demonstrates many of the key problems and dangers. Clearly safety is of utmost importance, and cardiac and cerebrovascular events appear to be the points of most concern.

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Chapter 36

Hemopure® (HBOC-201, Hemoglobin Glutamer-250 (Bovine)): Preclinical Studies

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INTRODUCTION

Hemopure® (HBOC-201, hemoglobin glutamer-250 (bovine)) is Biopure's third-generation product belonging to a new class of biologic oxygen therapeutics, hemoglobin-based oxygen carriers (HBOC). HBOC-201 was initially developed as a replacement for allogeneic red blood cells (RBCs) in patients undergoing surgery. The most important pharmacologic property of HBOC-201 is its ability to transport oxygen efficiently by promoting both convective supply and diffusive oxygen delivery.

The development of HBOC-201 has included completion of numerous pharmacology studies, not only with HBOC-201 but also with Biopure's first- and second-generation preparations, which had somewhat different characteristics. These formulations have included Hemopure 1 Solution (H1S, Polymerized Bovine Hemoglobin) and Hemopure 2 Solution (HBOC-301, Oxyglobin®) among others. The composition and properties of the various formulations are listed in Table 36.1. H1S contained 50 per cent glutaraldehyde polymerized hemoglobin and required frozen storage. The properties of Oxyglobin are similar to those of HBOC-201, but Oxyglobin's average molecular weight (200 kDa) is lower. Both Oxyglobin and HBOC-201 have been modified and formulated during manufacture to provide for room temperature stability for extended storage (3 years, 3–30°C). The reduction in tetrameric hemoglobin as well as the stabilization has resulted in a formulation with fewer side effects than earlier preparations. However, since HBOC-201 has oxygen transport capabilities similar to the earlier preparations, it can be expected that the tissue oxygenation effects seen with those formulations are applicable to HBOC-201 and therefore certain studies with the earlier formulations have been included in this discussion.

PRODUCT CHARACTERISTICS

HBOC-201 is a sterile, ultrapurified, pyrogen-free solution of polymerized bovine hemoglobin. It has been formulated at a concentration of 13 g/dl in a balanced electrolyte solution at a pH between 7.6 and 7.9 (Table 36.1). The product consists of a family of hemoglobin polymers mostly ranging in molecular weight from 130 to 500 kDa (average of 250 kDa), and has a viscosity of 1.3 cPs. By comparison, whole blood has a viscosity of approximately 4 cPs and saline a viscosity of 1 cPs. HBOC-201 is isoncotic and isosmotic, and has an intravascular half-life in humans of approximately 19 hours. Native hemoglobin has a *P*50 of approximately

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Table 36.1 Composition and properties of Biopure HBOC formulations

	HBOC-201 (Hemopure)	HBOC-301 (Oxyglobin)	HS1-2	HS1
Hemoglobin concentration (g/dl)	12–14	12–14	9–13	9–13
Methemoglobin (%)	<10	<10	<15	<15
*P*50 (mmHg)	40	40	25	17
pH	7.6–7.9	7.6–7.9	7.6–7.9	7.6–7.9
Endotoxin (EU/ml)	<0.5	<0.5	<0.5	<0.5
Phospholipid (nmol/ml)	<3	<3	<3	<3
Colloid oncotic pressure (mmHg)	25	42	49	60
Osmolarity (mOsm/kg)	300	300	300	290
Tetrameric hemoglobin (stabilized and unstabilized) (%)	$\leqslant 2$	$\leqslant 35$	$\leqslant 40$	$\leqslant 50$
Average molecular weight (kDa)	250	200	175	150
Viscosity (centipoise at 37°C)	1.3	1.3	NA	NA
Half-life in humans/animals (h)	19	18–43*	NA	15
Sodium lactate	27	27	0	0
Free gluteraldehyde (ug/dl)	<3.5	<3.5	<3.5	<3.5
Storage conditions (°C)	2–30	2–30	-20	-20

*Dose dependent; NA = not available.

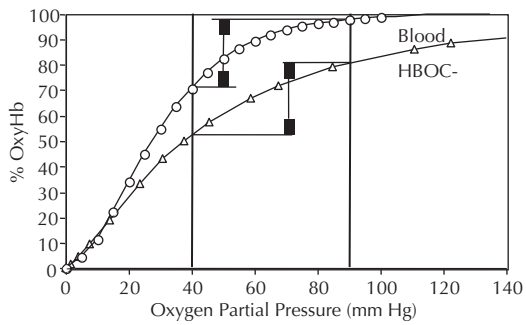

Figure 36.1 Oxygen equilibrium curves for human hemoglobin and HBOC-201 as measured by a Hemox Analyzer (TCS Scientific Corporation, New Hope, PA), at 37°C, pH 7.4. The horizontal lines intersecting the curves at the physiological PO_2 limits define the oxygen-delivery capacity.

27 mmHg, while BOC-201 has a lower oxygen affinity (*P*50 = 40 ± 6 mmHg; Figure 36.1). When stored as a solution under ambient temperature conditions between 2° and 30°C, HBOC-201 is stable for 3 years (Figure 36.2).

HBOC-201 can be administered immediately. It is compatible with all blood types; there is no time delay in waiting for results of blood typing, testing or cross-matching. It does not require warming or reconstitution prior to administration. It requires no specialized blood administration filters; it can be administered through a standard intravenous line. It transports oxygen immediately upon administration and under ambient conditions (i.e., works with room air, and does not require an oxygen mask). HBOC-201 is ultrapurified through a patented pharmaceutical manufacturing process demonstrated to remove or inactivate potential contaminants, including plasma proteins, RBC stroma, endotoxin and infectious agents (e.g., viruses, bacteria and BSE agents), to a purity of >99 per cent. HBOC-201 has the potential additional advantage of transporting oxygen in the plasma phase of blood, at low blood pressures, and through constricted or partially blocked blood vessels via the movement of plasma to areas of the body where RBC flow is restricted.

PHARMACOLOGICAL ACTION

The pharmacodynamics of HBOC-201 is primarily associated with oxygen delivery and, although it is also a colloidal protein solution, HBOC-201 enhances oxygenation by promoting both

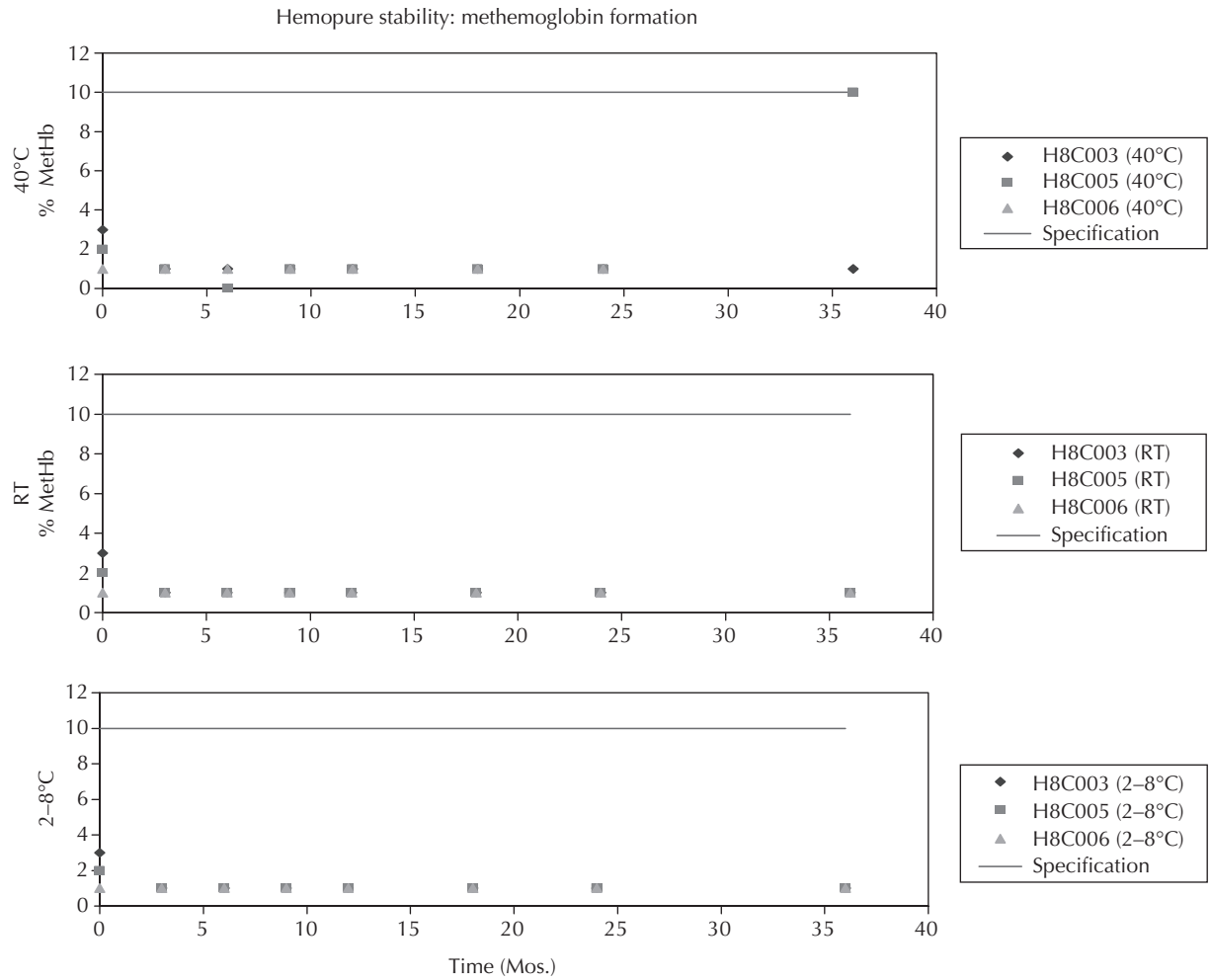

Figure 36.2 Percentage methemoglobin (% metHb) as a function of time for three lots of HBOC-201 at 37°C (75* humidity). The specification is no more than 10 per cent. The solid lines are linear regression fits.

convective and diffusive oxygen transport. The pharmacodynamic profile includes:

- increased rate of oxygen uptake and release (demonstrated *in vitro*)
- increased tissue oxygenation associated with increased oxygen extraction ratio
- eliminated and/or reduced tissue hypoxia caused by vessel stenosis and occlusion
- efficacy of oxygen transport evidenced in animal models of tumor oxygenation, hemorrhagic shock trauma, and veterinary use of Oxyglobin.

HBOC-201 promotes hemodynamic and metabolic stability, and has been shown to treat the signs and symptoms of anemia and to reduce tissue hypoxia in models of either extended cardiac arrest or myocardial infarction. Effects of earlier HBOC solutions on smooth muscle-mediated responses *in vivo* appear to have been attenuated by the reduction of the low molecular weight hemoglobin components in newer formulations (HBOC-201).

Enhanced oxygen transport: *in vitro* oxygen flux studies

In contrast to the hemoglobin in RBCs, HBOC-201 is uniformly distributed in the plasma phase of blood, impacting fundamental oxygen transport processes. The low solubility of oxygen in the plasma phase of erythrocyte suspensions generates large diffusional resistance. However, when added to plasma containing RBCs, HBOC-201 reduces this resistance and facilitates the off-loading of oxygen from erythrocytes (Page *et al.*, 1998a, 1998b). Furthermore, the lower oxygen

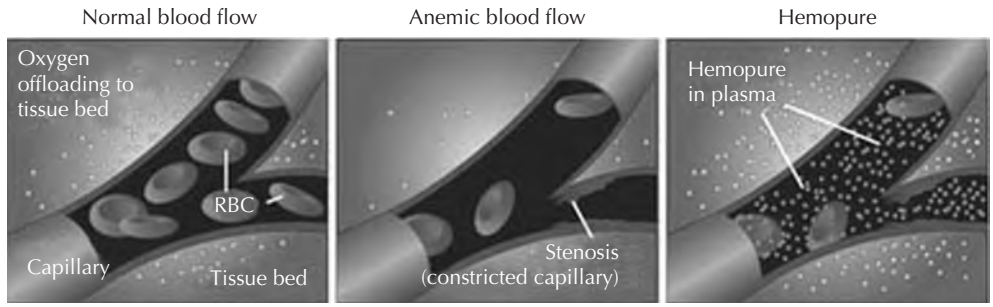

Figure 36.3 Because HBOC-201 circulates freely in plasma, and is smaller, has lower viscosity (resistance to flow) and can more readily release oxygen to tissues than red blood cells, it can carry oxygen at low blood pressure and through constricted or partially blocked blood vessels to areas of the body that red blood cells cannot reach due to their larger size. See color plate 15.

affinity of HBOC-201 compared to native RBC hemoglobin increases the tendency to off-load oxygen to tissues (Figure 36.3).

Page *et al*. developed and validated a mathematical simulation model (1998a) based on experimental data from *in vitro* capillary studies (1998b). This model was used to analyze the oxygen flux of various HBOC solutions, RBCs, and mixtures of the two in model capillaries. The degree of saturation of hemoglobin by oxygen was examined as a function of the residence time in an artificial capillary. HBOC was more efficacious than RBC suspensions in uptake and release of oxygen. Increasing the HBOC content of mixtures of HBOC and RBCs, or HBOC alone, resulted in an increase in the efficacy of oxygen transport (off-loading). In mixtures of RBCs and HBOC, when HBOC content reached 50 per cent the mixture had the equivalent oxygen transport efficacy of HBOC solution alone. These data suggest that HBOC more effectively transports oxygen than RBCs and, when added to RBCs, can increase the efficacy of RBC oxygen transport. These observations may explain, in part, the ability of Biopure's HBOC formulations to increase oxygen extraction and tissue oxygenation.

Persistent increases in oxygen extraction in the presence of HBOC may be indicative of improved oxygenation compared to RBCs alone. The observed decrease in cardiac output following administration of HBOCs may be an autoregulatory response to the increased efficiency of oxygen transport to tissues and increased oxygen extraction. These important properties of HBOCs are illustrated in this review of the published *in vivo* studies with the various HBOC formulations produced by Biopure.

PRECLINICAL STUDIES OF TISSUE OXYGENATION AND TRAUMA

The development of HBOC-201 has included completion of over 250 studies, many of which have been described in previous reviews (Light *et al*., 1998; Pearce and Gawryl, 1998). These studies demonstrated the efficacy of the various hemoglobin preparations in oxygenating tissues and stabilizing hemodynamics, sustaining life after complete exchange transfusion, resuscitation from hemorrhagic shock, hemodynamic support of animals with septic shock, and resuscitation following cardiac arrest, and characterized their effects on renal function. Furthermore, in assessing safety, these studies demonstrated that HBOC-201 produced few side effects: an increase in mean arterial blood pressure (MAP) (10–20 mmHg), gastrointestinal signs (vomiting, diarrhea), increases in serum enzyme activities (aspartate aminotransferase, lipase) not associated with clinical disease, and skin and/or scleral discoloration. A summary of the more recent animal studies is included below.

Tissue oxygenation

Several studies reviewed by Pearce and Gawryl (1998) directly demonstrated increased tissue oxygenation at higher oxygen extraction ratios and lower cardiac outputs after treatment with HBOC- 201 compared to RBCs. This hemodynamic phenomenon relates to the distribution of extracellular hemoglobin in the plasma phase of blood. More recent studies supporting these conclusions are described below.

Arterial stenosis and occlusion

The presence of HBOC in the plasma phase of blood results in a global distribution of oxygen-carrying hemoglobin in the circulation and contributes to the overall increase in oxygen extraction. Theoretically, the plasma phase distribution is of benefit when tissue injury or disease limit or prevent the flow of RBCs. Thus, the effect of HBOC in various animal models of partial to complete vessel occlusion has been studied to test this hypothesis.

The first study to demonstrate the potential benefit of HBOC-201 in partial vascular occlusion was that of Horn *et al*. (1997) in a canine model of arterial stenosis. Fourteen dogs underwent isovolemic hemodilution with lactated Ringer's (RL) to a hematocrit of 25 per cent, after which a 95 per cent artificial stenosis of the popliteal artery was experimentally induced. The animals were randomized to receive either 50 ml HBOC-201 or 200 ml 6 per cent hetastarch (HES). In both groups, oxygen delivery and oxygen consumption of the muscle decreased in parallel with decreasing blood flow following arterial stenosis. During stenosis, tissue PO_2, as measured by an Eppendorf polarographic needle microelectrode, was decreased in both groups when compared with baseline ($P < 0.001$). Following treatment, tissue PO_2 remained low in the HES group but returned nearly to baseline with HBOC-201 ($P < 0.001$). Similarly, the oxygen extraction ratio increased after infusion of HBOC-201 and was higher when compared with HES-treated animals ($P < 0.05$). With the exception of higher MAP and mean pulmonary artery pressure in HBOC-201 treated animals, hemodynamics did not differ between the two treatment groups. In contrast with HES infusion, administration of HBOC-201 resulted in the restoration of baseline skeletal-muscle tissue oxygen tensions during nearly complete arterial stenosis. This may be due to the ability of HBOC-bound oxygen to reach post-stenotic tissues via reduced plasma flow by the 95 per cent occlusion, as well as increased oxygen extraction despite the RBC flow being blocked.

One of the more important potential applications of HBOC-201 in cardiovascular disease may be in the treatment of ischemia due to coronary vessel occlusion. Standl *et al*. (1999) investigated the effect of 0.6 g/kg HBOC-201 or RL on tissue oxygenation in the heart of dogs during sustained normovolemic hemodilution (Hb $\leqslant$ 7.5 g/dl) and acute 90 per cent stenosis of the left anterior descending coronary artery (LAD). Cardiac muscle oxygenation was measured using a flexible microelectrode in the area supplied by the LAD. Following administration of RL or HBOC-201, before 90 per cent stenosis of the LAD, cardiac tissue oxygen levels distal to the occlusion site decreased from 21 ± 6 mmHg to 7 ± 6 mmHg in the RL treated group but were unchanged (18 ± 7 mmHg) in the HBOC-201 group. If HBOC-201 was administered after establishment of the stenosis, tissue oxygen levels were partially restored (23 ± 7 mmHg before stenosis and 15 ± 5 mmHg after stenosis). These results are similar to the earlier observations in canine muscle reported by Horn *et al*. (1997), where the presence of HBOC-201 in the plasma phase antagonized the ischemia that developed following 95 per cent vascular occlusion.

The beneficial effect of HBOC-201 may extend beyond the ability to perfuse and oxygenate tissue distal to partial occlusions. Strange *et al*. (2000) investigated the potential cardioprotective effects of HBOC-201 in a canine model of complete vascular occlusion of the LAD. HBOC-201 (equivalent to 10 per cent total blood volume) was infused just prior to a 90-minute complete occlusion of the coronary artery, after which reperfusion was established for 4.5 hours. Histological analysis of infarct size showed a greater than 55 per cent reduction as a result of HBOC-201 infusion compared with a vehicle control (0.9 per cent saline) ($P < 0.01$). Consistent with the decreased infarct size, neutrophil infiltration was also significantly ($P < 0.01$) reduced in the HBOC-201 group. Neutrophil accumulation into a previously ischemic zone is considered a hallmark of reperfusion injury following ischemia. Animals treated with HBOC-201 did not show the hemodynamic instability and arrhythmias that were characteristic of the control group and plasma levels of creatinine kinase and Troponin I were lower, consistent with the protective effect of HBOC-201 on cardiac muscle ($P < 0.05$).

The presence of HBOC-201 in the circulation compensates, in part, for the loss of adequate direct perfusion of cardiac muscle, and may involve enhanced transport of oxygen via collateral vessels. These data further suggest that HBOC-201 augmentation of oxygen transport may counteract the severe tissue hypoxia and injury that occur with complete vascular blockage.

Tumor oxygenation

Teicher and colleagues performed a series of studies to determine whether HBOCs increase PO_2 in

hypoxic tumors and, subsequently, if this enhanced tumor oxygenation would result in increased sensitivity to radiation and chemotherapy. Initial data (Teicher *et al.*, 1991, 1992a, 1992b, 1993) were generated from studies performed with HS1-2 (a variant of H1S with 40 per cent rather than 50 per cent unpolymerized hemoglobin) and later confirmed in the same models with HBOC-201 (Robinson *et al.*, 1995; Teicher *et al.*, 1995a, 1995b). Tumor PO_2 levels were measured directly in rats breathing either normal air (21% O_2) or carbogen (95% O_2/5% CO_2) using a polarographic needle microelectrode. Tumor growth delay was also measured following chemotherapy or the combination of chemotherapy and fractionated radiation treatment. In the five studies in which either single dose radiation or fractionated radiation treatment over 5 days or both were employed, HBOC increased tumor oxygenation and enhanced the irradiation response measured in terms of tumor growth delay and survival time (Robinson *et al.*, 1995; Teicher *et al.*, 1991, 1992a, 1992b, 1995a, 1995b). The radiation response was seen in mammary adenocarcinoma 13672, 9L gliosarcoma and FSaIIC Lewis lung carcinoma (fibrosarcoma) (Robinson *et al.*, 1995; Teicher *et al.*, 1993, 1995b). The addition of carbogen breathing to treatment with HBOC resulted in an increase in tumor PO_2 values and decreased the hypoxic fraction (per cent PO_2 readings $\leqslant$ 5 mmHg) of cells in tumors compared to HBOC alone (Robinson *et al.*, 1995; Teicher *et al.*, 1991, 1995a, 1995b). These changes were associated with increases in tumor growth delay and animal survival. Similar findings have been obtained when HBOC and HBOC/carbogen have been used in combination with alkylating chemotherapeutic agents (Teicher *et al.*, 1991, 1992a, 1992b, 1995a).

Collectively, the results of these studies suggest that infusion of Biopure's HBOC formulations produced increased tumor PO_2 levels and an associated decrease in the hypoxic fraction of tumors. In almost all treatment paradigms investigated, the increased tumor oxygenation corresponded with an increase in tumor growth delay. These studies provide further evidence of enhancement of tissue oxygenation by Biopure's HBOC formulations.

Trauma, hemorrhage and/or shock

Initial studies using hemodilution (Pearce and Gawryl, 1998) demonstrated the efficacy of various formulations of bovine HBOCs to satisfy oxygen demands under normotensive conditions without untoward effects. These early studies demonstrated that HBOC-201 could be used as an 'oxygen bridge', supplying oxygen needs until blood cells regenerated. The results of those studies provided the background for experiments assessing the use of HBOCs in the treatment of hypovolemic trauma with hypoperfusion.

Several studies (below) have assessed the efficacy of HBOC-201 in both acute and survival models of controlled or uncontrolled hemorrhage with severe organ injury and lethal whole body trauma, as well as acute and survival studies utilizing hypotensive resuscitation.

Controlled hemorrhage models

Studies with early HBOC formulations (Pearce and Gawryl, 1998) involved animal models of controlled hemorrhage and extended hypovolemia. A typical design for these models was the controlled removal of blood from a resected vessel or from an intravenous or intra-arterial catheter and maintenance of the resulting hypovolemic and hypotensive state for a predetermined time. These early studies demonstrated that resuscitation with HBOC restored hemodynamics, corrected acidosis to an extent comparable to RBCs, and supported survival equal to RBCs without causing any important acute adverse cardiopulmonary effects or organ dysfunction.

Uncontrolled hemorrhage models

Clinically, hemorrhage is often uncontrolled and associated with tissue injury. In standard prehospital care, normal saline or RL solution is administered to hypotensive trauma victims with the objective of delaying exsanguinating cardiac arrest. Several experiments have evaluated the use of HBOC-201 to treat severe, continuous, uncontrolled hemorrhage with associated liver injury modeling a patient with blunt abdominal trauma and uncontrolled hemorrhage in a prehospital setting.

Manning *et al.* (2000) developed a novel swine model of blunt-trauma liver injury with uncontrolled hemorrhage, shock and death. Liver injury was produced by partial resection of each of the four liver lobes. Nine minutes after the onset of bleeding, swine were randomized to receive approximately 10 ml/kg per minute of intravenous RL ($n = 10$) or HBOC-201 ($n = 7$) to obtain and maintain an MAP of 60 mmHg or until

hemodynamic collapse while bleeding continued for 2 hours. All animals were initially resuscitated successfully. However, the 2-hour survival was 1/10 with RL, and 7/7 with HBOC-201 ($P = 0.0004$). Nine swine treated with RL experienced cardiovascular collapse at 36 ± 10 minutes. Thirty minutes after resuscitation, lactate levels were significantly ($P < 0.05$) lower in HBOC-201 treated animals (12 ± 2 mmol/l) versus controls (18 ± 3 mmol/l). The severity of this model is reflected by the low hematocrit levels (<1 per cent) in the majority of treated and control animals. HBOC-201 infusion stabilized hemodynamics. The low volume requirement for HBOC-201 was reflected by the lower infusion rate for HBOC-201 treated animals (2.6 ml/kg per minute) versus the infusion rate for RL treated animals (10.8 ml/kg per minute) and is likely to prove useful in the pre-hospital-care environment, particularly when extreme transport times are required or in battlefield situations where resuscitative fluid availability is restricted by logistical constraints.

Katz *et al*. (2002) used the same exsanguinating liver injury model to assess survivability with HBOC-201. Swine underwent a liver crush, laceration, and 50 ml/kg initial blood loss. The liver continued to bleed at 3 ml/kg per minute during the resuscitation phase. Withholding fluid resuscitation (NF, n = 6) or resuscitation with HES (n = 8) were fatal in this model, while all HBOC-201 (n = 8) swine survived 24 hours and seven of eight survived 96 hours with good functional recovery. The investigators concluded that HBOC resuscitation during profuse liver bleeding and traumatic shock enhanced survival with good physiological outcome in swine.

Aggressive use of HBOC-201 during resuscitation from severe hemorrhage may have limitations, however. When large volumes of HBOC-201 were administered rapidly (6 ml/kg per minute to animals) with severe volume depletion in the study (Katz, 2000), blood pressure and pulmonary artery pressure increased. These changes did not compromise pulmonary function or survival, and future studies will assess their effect on the potential to increase bleeding.

Model of lethal whole body trauma

Severe trauma is often associated with intravascular coagulation, interstitial edema and release of toxic humoral mediators, leading to severe hypotension and multiple organ injury. Thus, Hayward and Lefer (1999) examined the effect of HBOC-201 infusions (5 per cent, 10 per cent, and 15 per cent of calculated blood volume) in a rat model of lethal traumatic shock. Anesthetized rats subjected to Noble–Collip drum trauma developed shock with marked hypotension, as well as splanchnic vascular endothelial dysfunction characterized by an impaired vasorelaxation response of the superior mesenteric artery (SMA) to endothelium-dependent vasodilators, and a four-fold increase in intestinal myeloperoxidase activity. Treatment of rats 10 minutes post-trauma with 10% HBOC-201 resulted in a two-fold increase ($P < 0.05$) in survival time from 108 ± 20 minutes in control animals to 228 ± 31 minutes in HBOC-201 animals. HBOC-201 also normalized MAP and produced a marked preservation of mesenteric vascular endothelial function. Treatment with HBOC-201 had no effect on neutrophil infiltration as indicated by an absence of change in ileal tissue content of the polymorphonuclear leukocyte enzyme, myeloperoxidase. Treatment with a vehicle control post-trauma did not result in any beneficial effects. These data suggest that infusion of HBOC-201 post-trauma normalizes systemic blood pressure and protects endothelial function.

Hypotensive resuscitation

The ability to provide adequate tissue oxygenation under conditions of low volume and pressure resuscitation can be of enormous value in battlefield or civilian casualty situations, particularly for far forward or remote locations where supplies are limited and delayed evacuation is expected. Several studies investigated the effects of HBOC-201 using animal models of low-volume or hypotensive resuscitation.

McNeil *et al*. (2001) evaluated the ability of HBOC-201 to restore tissue perfusion under conditions of hypotensive resuscitation in a porcine model of severe hemorrhage to a MAP of 30 mmHg. Animals were maintained at a MAP of 35 mmHg for 45 minutes by continuous hemorrhage and subsequently resuscitated with HBOC-201, RL, or RL plus blood to a MAP of 60–80 mmHg. Animals were monitored for 4 hours post-resuscitation.

As demonstrated by measurements of base excess, pH and lactate levels, low-volume (as compared to LR or LR plus blood) and low-pressure resuscitation with HBOC-201 after controlled hemorrhage in swine provided sufficient tissue perfusion and oxygen delivery to reverse anaerobic metabolism in the presence of continued hypotension, hypovolemia and low cardiac

Table 36.2 Resuscitation volumes after hypotensive resuscitation with HBOC-201

Group	Resuscitation volume (ml)
HBOC-201 @ 60 mmHg	463 ± 57
LR @ 80 mmHg	16 358 ± 2571
LR + blood @ 80 mmHg	4777 ± 260
P value (ANOVA)	<0.001

output (Table 36.2). Similar results were seen when this study was repeated (York *et al*., 2003) and animals allowed to survive. Hypotensive resuscitation with HBOC-201 provided adequate tissue oxygenation for survival in this porcine model of controlled hemorrhage.

HBOC-201 enhances oxygen tension and tissue oxygenation when traditional interpretation predicts otherwise. The potential adverse effects of hypovolemia, hypotension and low cardiac output are all compensated for by the oxygen transport characteristics of HBOC-201, as demonstrated by measures of blood lactate, pH, jejunal oximetry and physiologic outcome following resuscitation and sustained survival over several days. Sampson *et al*. (2003) compared 7.5% hypertonic saline (HS), hypertonic saline 7.5%/6% dextran-70 (HSD), 6% pentastarch, 6% HES, or HBOC-201 with RL and no resuscitation controls in a swine model of controlled hemorrhagic shock. After 45 minutes of shock, animals were resuscitated to and maintained at a MAP of 60 mmHg for 4 hours. Death occurred in five of six animals in the no-resuscitation control group, six of six in the HS group, and one animal in the HSD group before completion of the study. HBOC-201 restored tissue oxygenation and reversed anaerobic metabolism at significantly lower volumes when compared to HTS, HSD, Pentastarch or HES.

Brain oxygenation during hemorrhagic shock

Outcomes after head injury, the leading cause of traumatic death in the US, are severely worsened in the presence of hypotension. HBOC-201 treatment of hypovolemia in patients with traumatic brain injury may lead to improved outcomes. Previous reports (Sadrzadeh *et al*., 1987; Regan and Panter, 1993, 1996) have suggested that hemoglobin solutions can be toxic to neurons *in vivo* and *in vitro* in culture. In some of these studies, many different preparations, from autologous blood to 'purified hemoglobin', were injected into the brain. Other studies focused on establishing the role of hemoglobin released from hemolyzed RBCs in the pathological vasospasm that follows subarachnoid hemorrhage rather than on examining the potential direct neurotoxic effects of hemoglobin. To what extent the reported effects of HBOC solutions relate to the vasoactivity of tetrameric hemoglobin, the direct neurotoxic effect of hemoglobin, or toxic effects of some other component of these preparations is not clear.

Two early studies by Waschke *et al*. (1993, 1994) demonstrated that near total replacement of blood by H1S satisfied the circulatory and metabolic demands of the normal rat brain. In a more recent study of brain tissue oxygenation, Manley *et al*. (2000) investigated the effects of resuscitation with HBOC-201 (in a swine model of pre-hospital small-volume resuscitation) on brain tissue oxygen tension, MAP and cardiac output (CO). MAP and CO decreased significantly with hemorrhage. Small volume resuscitation with HBOC-201 restored and maintained cerebral oxygenation, MAP, and CO following severe hemorrhagic shock in swine. In addition, in a double-blind study using a similar swine model of hemorrhagic shock, Manley and coworkers (2002) compared the effects of hypertonic saline dextran (HSD), RL, and HBOC-201 on brain oxygenation. Resuscitation with HBOC-201 provided more efficacious and durable improvement in brain oxygen and cerebral perfusion pressure. However, in the same model Knudson *et al*. (2003) failed to show improvement in liver or deltoid muscle oxygenation with HBOC-201 (and 100% O_2) in comparison with RL or HSD (and 100% O_2). MAP and systolic blood pressure were stabilized more effectively in HBOC-201 pigs, while cardiac output was highest in HSD-treated pigs.

These results, indicating that HBOC-201 provides adequate brain tissue oxygenation and promotes long-term survival, are supported by studies assessing the effects of HBOC-201 on neurons in cell culture. Ortegon *et al*. (2002) compared the effects of incubating HBOC-201 and purified human hemoglobin (hHb, Sigma) (0.02, 0.2, 2.0 and 6.5 g/dl) with rat fetal neural cells in culture for 24 hours. Neural cells exposed to HBOC-201 did not lyse, and maintained levels of proliferation and metabolism similar to controls. However, cultures exposed to hHb ($\geqslant$0.2 g/dl) demonstrated significantly decreased proliferation, decreased metabolic activity, and increased cell lysis when compared with controls ($P < 0.05$). Neural cells exposed to HBOC-201 in culture were able to continue sustained metabolic activity and normal

proliferation with no evidence of neurolysis, suggesting that HBOC-201 does not display the toxic characteristics of hHb.

CARDIOVASCULAR STUDIES

Early cardiovascular studies examined the effects of hemodilution and complete blood replacement on cardiovascular function and hemodynamics. Slanetz *et al*. (1994) showed that HS1-2, even when used to replace 80 per cent or more of the RBC mass, was effective in oxygen transport and was able to meet the oxygen demands during surgical stress and recovery. Vlahakes *et al*. (1990), in a model of massive blood replacement, demonstrated that HS1 supported oxygen consumption at baseline levels and long-term survival with rapid resynthesis of erythrocytes. In a similar model of near complete blood replacement, using a more polymerized formulation, HS1-2 (40 vs 50% tetrameric hemoglobin), Lee *et al*. (1995) demonstrated increases in systemic (SVR) and pulmonary vascular resistances (PVR), 44 per cent and 200 per cent, a decrease in cardiac output, but with a persistent elevation of oxygen extraction ratio. In contrast to animals hemodiluted with HS1-2, the oxygen extraction ratio of the control animals steadily increased with continuing hemodilution and did not stabilize. The impact of the increase in oxygen extraction with HS1-2 was illustrated in studies performed by Standl *et al.* (1997). Using HBOC-201 in a canine model of complete blood exchange, Standl *et al.* (1997) showed a similar increase in SVR (60 per cent) and a sustained increase in oxygen extraction ratio, but no difference in PVR following infusion. Despite the increase in SVR and consistent with the increase in oxygen extraction, there was a significant increase in tissue PO_2 (50 mmHg) with HBOC-201 infusion compared with hetastarch (13.6 mmHg) as measured by a polarographic needle microelectrode (Eppendorf).

Lee *et al.* (1995) also observed significant increases in plasma methemoglobin 24 hours (4.0 ± 0.7 per cent per hour) following administration of HS1-2, and attributed it to the tetrameric form of hemoglobin. The development of HBOC-201 involved the reduction of tetrameric hemoglobin from 40 per cent in HS1-2 to ~2 per cent in HBOC-201. Methemoglobin data from clinical studies (see Chapter 37) demonstrate a lower rate of methemoglobin formation (~0.08 per cent per hour) consistent with the near complete removal of tetrameric hemoglobin.

HEMODILUTION AND COMPLETE BLOOD REPLACEMENT

Standl *et al*. (1997) studied the effect of complete isovolemic hemodilution with HBOC-201, in comparison to HES, on hemodynamics, oxygen transport capacity and tissue oxygenation in a canine model. Stepwise hemodilution with RL was used to decrease hematocrit to 20 per cent, followed by progressive blood exchange with 6% HES or HBOC-201 until hematocrit target levels of 15 per cent, 10 per cent and 5 per cent or less were obtained. In HES-treated animals, heart rate, cardiac output and blood flow increased during hemodilution ($P < 0.05$); systemic vascular resistance, arterial and venous oxygen content, arteriovenous oxygen difference and skeletal muscle tissue oxygenation decreased ($P < 0.05$). In HBOC-201 treated animals, heart rate, cardiac output and arterial blood flow decreased ($P < 0.05$) but arterial oxygen content did not change. Arteriovenous oxygen difference, oxygen extraction ratio, systemic vascular resistance and skeletal muscle tissue oxygenation increased ($P < 0.05$) with pro- gressive hemodilution with a maximal contribution of HBOC-201 to arterial oxygen content of 82 per cent. In spite of a higher final hematocrit of 5 per cent in HES-treated animals (versus 2 per cent in HBOC-201 treated animals), final muscular oxygen uptake (4.7 ± 4 vs 10.1 ± 2 ml/min) and mean tissue PO_2 (11.8 ± 2.3 vs 51.1 ± 2.9 mmHg) were lower in HES-treated animals than in HBOC-201 treated animals. At a final hematocrit of 2 per cent, HBOC-201 also provided hemodynamic stability for at least 1 hour, while HES-treated animals had to be sacrificed at a hematocrit of 5 per cent due to severe cardiovascular decompensation. Almost complete blood exchange under stable hemodynamic conditions was associated with optimal tissue oxygenation in animals with HBOC-201 exchange. In contrast to HES-treated animals, oxygen delivery was maintained by increased extraction of oxygen rather than by increases in cardiac output.

CARDIAC FUNCTION, OUTPUT AND METABOLISM

The effects of HBOC-301 on ventricular systolic function and ventricular vascular coupling in normal dogs were investigated by Muir *et al*. (2000). Dogs received either 6% dextran 70 (control) or HBOC-301 at 15 ml/kg. Control animals showed no significant differences during

the experiment in heart rate, stroke volume, cardiac output; systolic, diastolic and MAP; systolic and diastolic left ventricular volume; stroke work, dP/dt_{max}; end systolic elastance (E_{es}); or effective arterial elastance (E_a). HBOC-301-treated animals exhibited no significant differences in heart rate, stroke volume, systolic and diastolic left ventricular volume and E_{es}. However, cardiac output, dP/dt_{max}, and stroke volume decreased, while systolic, diastolic and MAP, systolic and diastolic left ventricular pressure and E_a increased. These results were consistent with the effect of hemoglobin on nitric oxide (NO). Hemoglobin has been known to oxidize and bind nitric oxide (NO), however, studies (Alayash *et al*., 2001) that measured NO-induced oxidation of HBOC-301 demonstrated a reduced rate constant ($13\,\mu M^{-1}s^{-1}$) compared to native bovine hemoglobin ($21\,\mu M^{-1}s^{-1}$) and chemically modified human hemoglobin solutions ($25–30\,\mu M^{-1}s^{-1}$). This two-fold reduction of the rate of oxidation may significantly reduce the potential effect on endothelial function and NO regulation of hemodynamics.

The reaction of hemoglobin with NO is also thought to account for the ability of hemoglobin solutions to block the inhibition of mitochondrial respiration in target cells by cytotoxic macrophages (Weinberg and Hibbs, 1977). Loke *et al*. (2000) studied the effect of HBOC-201 on mitochondrial tissue respiration, oxygen consumption, and substrate utilization in the canine heart. Dogs were infused with HBOC-201 (equivalent to 20 per cent blood volume) or angiotensin II. HBOC-201 caused an increase in left ventricular systolic pressure, MAP, and mean coronary blood flow at 15 minutes and 120 minutes; late diastolic coronary resistance (LDCR) and left ventricular dP/dt_{max} were not affected. HBOC-201 had no effect on arterial or coronary sinus blood pH or partial pressure of carbon dioxide (PCO_2) at any time point. However, coronary sinus PO_2 was significantly reduced after HBOC-201 infusion, indicating an increase in oxygen extraction fraction by the heart. Like HBOC-201, angiotensin II increased left ventricular pressure and MAP at 60 and 120 minutes, respectively. However, in contrast to HBOC-201, angiotensin II did not increase coronary blood flow but significantly increased LDCR at 60 and 120 minutes. HBOC-201 administration caused sustained increases in left ventricular systolic and arterial pressures, and left circumflex coronary artery blood flow. In addition, 15 minutes after HBOC-201 infusion, myocardial oxygen consumption more than doubled, from 7.2 ± 0.8 to 15 ± 1.8 ml O_2/min ($P < 0.01$), myocardial free fatty acid consumption decreased from 14 ± 1 to $4.5 \pm 2.2\,\mu$Eq/min, lactate consumption increased from 19 ± 6 to $69 \pm 10\,\mu$mol/min, and consumption of glucose increased from 1.0 ± 0.5 to 10 ± 3 mg/min (all P values < 0.05). These metabolic changes were not seen with angiotensin II, suggesting that they may be independent of the hemodynamic changes. The authors concluded that administration of HBOC increases coronary blood flow and oxygen consumption, and shifts cardiac metabolism from using free fatty acid to using lactate and glucose in conscious dogs at rest. The yield of ATP per molecule of oxygen is 3.17 for glucose and 2.83 for free fatty acids. These data suggest that in the presence of HBOC, metabolism in the heart shifts to utilization of glucose and the observed increase in oxygen consumption may be associated with greater availability of ATP for the heart.

A series of studies (Pearce and Gawryl, 1998) suggests that polymerized bovine hemoglobin can be used to restore cardiac function after periods of extended cardiac arrest. More recently, Manning *et al*. (2001) evaluated the acute cardiovascular and metabolic effects of selective aortic perfusion (SAAP) with HBOC-201 in a swine model of exsanguinating cardiac arrest. Partial resection of four liver lobes rapidly led to hemorrhagic shock (blood loss of 40 ml/kg) and subsequent cardiac arrest within 10–13 minutes. At 15 minutes, swine received SAAP with either oxygenated RL or oxygenated HBOC-201 until return of spontaneous circulation (ROSC) with a MAP of 60 mmHg was achieved. Epinephrine (0.005 mg/kg) was given intra-aortic every 30 seconds as needed to promote ROSC beginning at 18 minutes after onset of liver injury (3 minutes after beginning SAAP). MAP, cardiac output, total blood loss and time of arrest were similar for both groups prior to SAAP therapy. In the HBOC-201 group, ROSC was achieved in six of six swine at 1.9 ± 0.3 minutes of SAAP, and none of these swine required epinephrine. In the RL group, none of six swine achieved ROSC prior to intra-aortic epinephrine administration and only two of six swine had brief (< 10 minutes) ROSC after intra-aortic epinephrine. One-hour survival occurred in five of six animals in the HBOC-201 group and none of six in the RL group ($P < 0.05$). SAAP with oxygenated HBOC-201 and perfusion of the aortic arch rapidly restored viable cardiovascular function after exsanguinating cardiac arrest in this swine model of liver injury with profound hemorrhagic shock.

POTENTIAL INDICATIONS FOR HBOC-201

Extracorporeal membrane oxygenation

A potential application for HBOC-201 is as an oxygen source in extracorporeal membrane oxygenation (ECMO). Before the advent of ECMO support, term and near-term neonates with cardiorespiratory failure experienced a mortality rate of $\geqslant 80$ per cent. With ECMO, survival rates are greater than 90 per cent for some diagnoses (York *et al*., 2002). Because neonates have such small blood volumes, it is necessary to prime the ECMO circuit with a source of oxygen – in most cases, packed RBCs.

York *et al*. (2002) studied HBOC-201 substitution for blood in ECMO in a healthy immature porcine model. Of ten piglets placed on venoarterial ECMO, four animals received blood-primed ECMO while six animals received HBOC-201-primed ECMO. All animals survived the 6-hour ECMO procedure. Post-priming volume was 176 ± 156 ml in the blood group. None of the animals in the HBOC-201 group required additional volume to maintain target flow during ECMO ($P < 0.05$). The study found that HBOC-201-primed ECMO in a healthy porcine model showed similar hemodynamics and equivalent oxygen-carrying capacity to blood-primed ECMO, despite delivering a significantly lower hematocrit. Post-priming volume requirement was decreased significantly in the HBOC-201 group.

SUMMARY

Preclinical studies in dogs, sheep and rats have established the fundamental pharmacological properties of HBOC-201. HBOC-201 on-loads and off-loads oxygen efficiently, thus increasing systemic oxygen transport and tissue oxygenation. HBOC-201 promotes hemodynamic and metabolic stability and eliminates or reduces tissue hypoxia due to poor perfusion. HBOC-201 has been shown to treat the signs and symptoms of anemia and to reduce tissue hypoxia in models of arterial stenosis, hemorrhagic shock, extended cardiac arrest and myocardial infarction. Effects on smooth muscle-mediated responses *in vivo* have been diminished by polymerization and fractionation to reduce low molecular weight hemoglobin components. There is no evidence of untoward effects on coronary circulation, cardiac function, neurons *in vivo* or culture, the microcirculation, endothelial viability or renal function. The pharmacologic profile of HBOC-201 predicts clinical efficacy and safety in a spectrum of physiologic and pathologic conditions especially in a wide variety of conditions where RBCs would not be practical.

EDITOR'S SUMMARY

HBOC-201 is a product whose raw material is bovine hemoglobin, highly purified by column chromatography and polymerized with glutaraldehyde. It has been in development for about two decades. Early formulations contained large amounts of unpolymerized hemoglobin ($\sim$50 per cent) and newer ones have all but eliminated this component ($\sim$2 per cent), following the consensus in the field that it is low molecular weight, unpolymerized hemoglobin that is toxic, especially to the kidney. The product enjoys the advantages of inexpensive raw material (cow blood) and the company has very cleverly formulated HBOC-201 as deoxyhemoglobin to increase its storage stability so that it can be used under potentially harsh environments such as faced by the military.

Preclinical animal studies were focused on demonstrating superiority in hemorrhage/ resuscitation models of a variety of types, mainly with excellent results. An important series of experiments using an artificial capillary system demonstrated the remarkable efficiency of oxygen transfer from cell-free hemoglobin. These studies showed unquestionably that HBOC-201 could oxygenate tissues; in fact, complete blood replacements were done in some experiments. However, the classic hemoglobin response was seen, particularly in earlier formulations: increased systemic and pulmonary pressures and resistances, decreased cardiac output and bradycardia.

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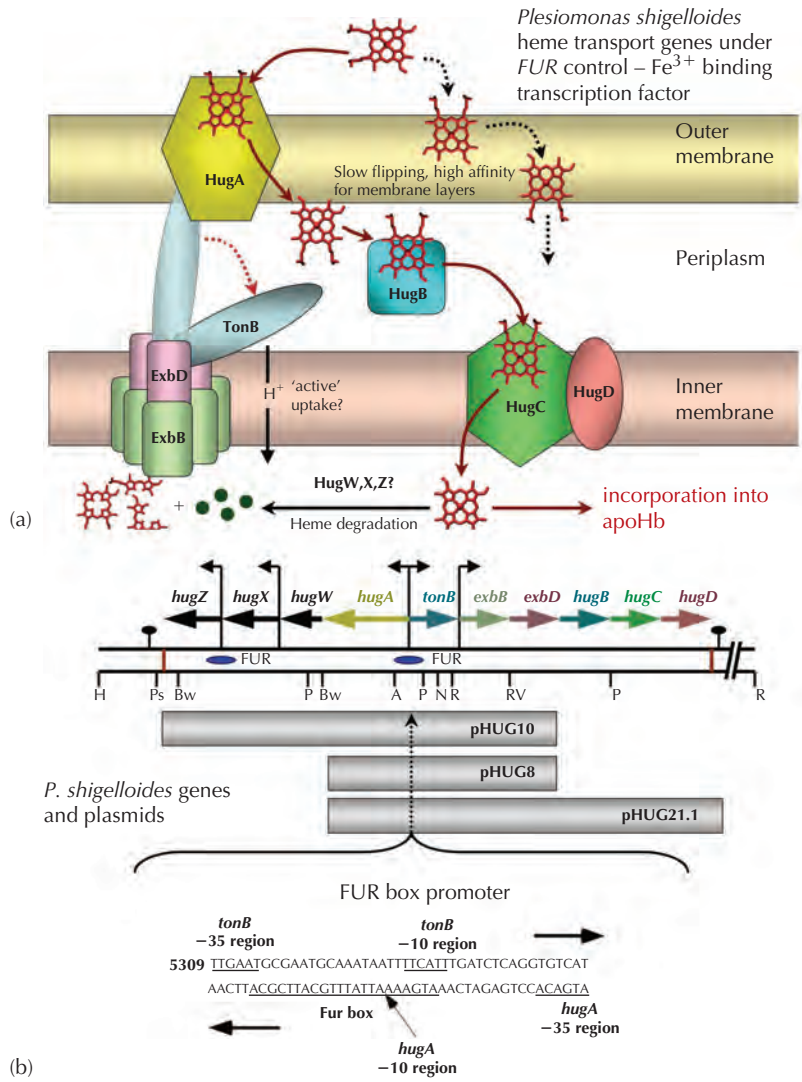

Plate 14 (a) Scheme for heme transport in *P. shigelloides* and related pathogens. The structural interpretation of the tonB system was taken from Seliger *et al*. (2001) and Postle and Kadner (2003), and the overall system from Stojiljkovic and Perkins-Balding (2002). Although hemin incorporation into the outer layers of phospholipids membranes is fast, non-facilitated flipping of the heme propionates is very slow. (b) Map of the *P. shigelloides* genes, FUR box and plasmids used for the co-expression experiments with rHb0.0 developed by Henderson (Henderson *et al*., 2001). See Fig. 31.10.

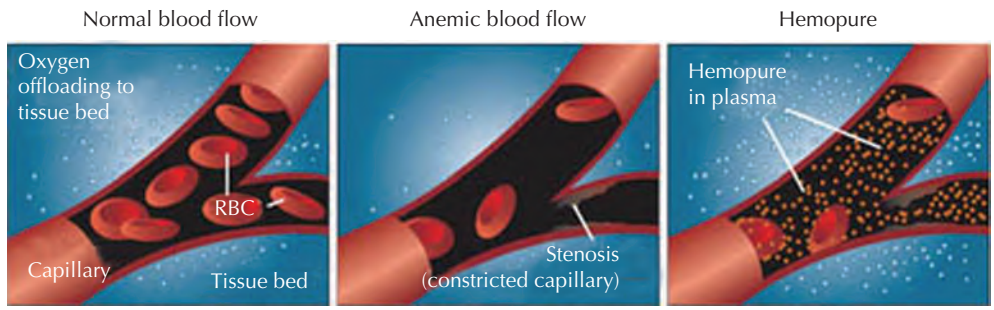

Plate 15 Because HBOC-201 circulates freely in plasma, and is smaller, has lower viscosity (resistance to flow) and can more readily release oxygen to tissues than red blood cells, it can carry oxygen at low blood pressure and through constricted or partially blocked blood vessels to areas of the body that red blood cells cannot reach due to their larger size. See Fig. 36.3.

Chapter 37

HBOC-201 (Hemoglobin Glutamer-250 (Bovine), Hemopure®): Clinical Studies

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INTRODUCTION

The safety and efficacy of HBOC-201 (Hemopure®, hemoglobin glutamer-250 (bovine)) (Biopure Corporation, Cambridge, MA) have been examined in 22 multinational clinical trials (Table 37.1) which enrolled a total of 1467 patients (806 received HBOC-201). The majority of these studies were conducted in the surgical setting, and included patients undergoing vascular, cardiac, hepatic, orthopedic, genitourinary and general surgical procedures. The clinical development of HBOC-201 has progressed conservatively, as both the dose of HBOC-201 and the interval over which it could be administered were increased in a stepwise fashion with each subsequent trial to provide an 'oxygen treatment bridge' to eliminate or reduce the need for red blood cell (RBC) transfusions. Three different dosing regimens have been investigated: fixed-gram doses, patient weight-adjusted doses, and 'unit' doses. In more recent dose-escalation studies, doses of up to 300 g (10 units) have been administered over a maximum 6-day treatment period.

Table 37.2 summarizes the clinical studies performed with HBOC-201 and the maximum doses administered.

Establishment of appropriate safety and efficacy endpoints for oxygen therapeutics has been challenging to the industry and debated for many years. Currently, avoidance of allogeneic RBC in patients undergoing surgery has been deemed an acceptable efficacy endpoint by the Food and Drug Administration. Furthermore, in surgical situations where blood is available, an oxygen therapeutic is considered safe if the rate and severity of adverse events are comparable to those seen with RBC.

This chapter will discuss published safety and efficacy studies performed only with HBOC-201. A Phase I safety study, performed with an earlier formulation that contained 50 per cent tetrameric hemoglobin and required frozen storage, was suspended after human volunteers experienced unacceptable gastrointestinal effects. Further development of that formulation was abandoned in favor of HBOC-201.

PHASE I STUDIES IN HUMAN VOLUNTEERS

Preclinical studies (see Chapter 36) demonstrated that HBOC-201 has the ability to enhance oxygen delivery and tissue utilization of oxygen, promote hemodynamic and metabolic stability, and maintain plasma volume in various animal models. These properties also were demonstrated in four Phase I studies of HBOC-201 in normal healthy volunteers in which subjects received doses up to 0.8 g hemoglobin from HBOC-201 (Hb)/kg using a model of 'surgical blood loss' (phlebotomy and volume replacement). The

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Table 37.1 Clinical pharmacology, pharmacokinetic, clinical efficacy and safety trials conducted in healthy volunteers and in surgical and non-surgical patients with HBOC-201

Primary objective (no. of centers)	Study design	Number of subjects treated	Treatment: dose (total g) and period	Age (years) Gender
Healthy Volunteer Studies (Phase I)				
*Safety, PK, and diffusion capacity (1)	R, SB, PG, SD, DE, Plac-con	HBOC-201 = 32 HSA = 23	HBOC: 3.3–65 g Hb HAS: equivalent volume Up to 4 h	18–45 100% male
*Safety, PK, and diffusion capacity (1)	R, SB, Plac-con, PG, SD, RE	HBOC-201 = 18 LR = 6	HBOC: 45 g Hb equivalent volume 45–90 min	18–45 50% male
*Safety, exercise tolerance (1)	R, SB, SD crossover	HBOC-201 = 6 Blood = 6	HBOC: 45 g Hb Blood: 150 g Hb 90 min	25–45 100% male
Immunology (1)	Open-label: up to three doses	HBOC-201 = 8	HBOC: 45 g Hb 90 min	18–45 100% male
Surgical patient studies (Phase I/II)				
*Abdominal aortic surgery ANH (1)	R, SB, PG, SD, DE	HBOC-201 = 19 HES = 20	HBOC: 0.4–1.2 g Hb/kg (28.5–97.8 g) HES: equivalent volume 30 min	44–75 74% male
*Liver resection surgery (1)	R, SB, PG, SD	HBOC-201 = 6 HES = 8	HBOC: 0.4 g HB/kg (22.4–36.4 g) HES: equivalent volume 30 min	25–70 64% male
Orthopedic surgery (1)	R, SB, PG, SD	HBOC-201 = 6 HES = 8	HBOC: 0.9 g Hb/kg (43.2–66.6 g) HES: equivalent volume 40 min	45–76 7% male
Radical prostatectomy (3)	R, SB, Plac-con, PG, SD, DE	HBOC-201 = 16 LR = 11	HBOC: 0.4–0.6 g/kg (0.5–45 g) LR: equivalent volume 90 min	48–73 100% male
Gynecological surgery (4)	R, SB, Plac-con, PG, SD, DE	HBOC-201 = 10 LR = 8	HBOC: 0.4–0.6 g/kg (23.4–45 g) LR: equivalent volume 60–90 min	24–73 100% female
Orthopedic surgery (4)	R, SB, Plac-con, PG, SD, DE	HBOC-201 = 13 LR = 10	HBOC: 0.4–0.6 g/kg (21.8–45 g) LR: equivalent volume 60–90 min	49–80 39% male
Urgent Ob/Gyn surgery (2)	R, SB, Plac-con, PG, SD, DE	HBOC-201 = 1 LS = 1	HBOC: 0.4–0.6 g/kg (27 g) LR: equivalent volume 30–90 min	31–37 100% female
*Non-cardiac surgery (6)	R, SB, Plac-con, PG, SD, DE	HBOC-201 = 55 LR = 26	HBOC: 0.6, 0.9, 1.2 g/kg (0.7–245 g) LR: equivalent volume 45–60 min	35–86 59% male
Non-cardiac surgery (6)	R, SB, Plac-con, PG, 2D, DE	HBOC-201 = 25 LR = 14	HBOC: 0.6 and 0.4 g/kg, 0.9 and 0.4 g/kg, 0.9 and 0.6 g/kg (0.7–166 g) LR: equivalent to volume 1st dose: 45–60 min, 2nd dose: 24 h later, 30–45 min	36–75 82% male
Surgical Patient Studies (Phase II)				
*Cardio-pulmonary bypass surgery (14)	R, DB, PG, RBC-con, MD	HBOC-201 = 50 RBC = 48	HBOC: 3 inf: (60–120 g) RBC: 3 inf: 1 unit each Up to 3 inf. over 72 h	44–82 62% male
*Abdominal aortic reconstruction (8)	R, SB, PG, RBC-con, MD	HBOC-201 = 48 RBC = 24	HBOC: 4 inf: (60–150 g) RBC: 4 inf: 1unit each 4 inf. postop. over 96 h	41–82 81% male
Non-cardiac surgery (3) (Military hospitals)	R, SB, Plac-con, PG, MD	HBOC-201 = 26 LR = 25	HBOC: 3 inf. (90–300 g) LR: 750–2500 ml Up to 3 inf. over 72 hr	24–83 84% male

(*Continued*)

Table 37.1 (*Continued*)

Surgical patient studies (Phase III)				
Non-cardiac surgery (21)	R, SB, PG, RBC-con, MD	HBOC-201 = 83 RBC = 77	HBOC: 6 inf: (60–210 g) RBC: 6 inf. 1 unit each 6 inf. periop. over 6 sd	21–86 56% male
Orthopedic surgery (46)	R, SB, PG, RBC-con, MD	HBOC-201 = 350 RBC = 338	HBOC: 9 inf. (60–300 g) RBC: 10 inf. 1 unit each	18–95 45% male
Non-surgical patient studies				
*Sickle cell not in crisis (1)	R, SB, Plac-con, PG, SD	HBOC-201 = 12 Saline = 7	HBOC: 0.2, 0.4, 0.6 g/kg (10.8–42.9 g) Saline: equivalent volume 30–60 min	19–47 84% male
Weaning from ventilation (1)	R, SB, Plac-con, PG, 2D	HBOC-201 = 1 LR = 0	HBOC: 0.6 g/kg each dose (90.6 g) LR: equivalent volume 2 doses 24 h apart	85 100% male
Sickle cell in crisis (5)	R, SB, Plac-con, PG	HBOC-201 = 12 LR = 7	HBOC: 0.6 then 0.3 g/kg (14.0–78.3 g) LR: equivalent volume 2 doses 24 h apart	7–48 79% male
Glioblastoma patients (1)	Open label	HBOC-201 = 9	HBOC: 0.5 g/kg 3 × wk for 6 wks	NA

*Published studies; R = randomized; SB = single blind; DB = double blind; PG = parallel group; SD = single-dose; 2D = two doses; MD = multiple-dose; Plac-con = placebo-controlled; RBC-con = red blood cell controlled; DE = dose-escalation.

Table 37.2 HBOC-201 clinical trials

No. of studies	Type	HBOC-201 patients (n)	Control patients (n)	Maximum total dose (g Hb)
4	Healthy volunteers	64	29	45–140
4	Non-surgery	34	14	43–1230
3	Surgery with ANH*	31	36	36–98
6	General surgery	120	70	27–245
1	Military surgery trial	26	25	300
4	Major surgery trials	531	487	120–300
Total: 22		806	661	27–1230

*ANH = acute normovolemic hemodilution.

results of these four studies were described in three publications.

Hughes *et al*. (1996a) studied the safety and pharmacokinetics of HBOC-201 in a randomized, single-blind, dose-escalation study in normal healthy men 18–45 years of age. Subjects underwent a 15 per cent blood volume phlebotomy, followed by volume replacement with lactated Ringer's (RL) (three times the volume of whole blood removed), and then infusion of either HBOC-201 (16.5, 24.1, 30.2, 38 or 45 g Hb) or RL (volume equal to HBOC-201 volume). There was a dose-dependent increase in plasma hemoglobin with peak concentrations of 1–2 g/dl at the 38 and 45 g doses. The plasma half-life at these doses was approximately 20 hours, the volume of distribution was 3–3.6 l of plasma, and the clearance was 0.11–0.13 l/h, indicating that the clearance of HBOC-201 was a linear first-order process. No hemoglobin was detected in the urine throughout the study. Whole blood methemoglobin increased by 1–2 per cent in the first 24 hours after HBOC-201 administration.

This study also examined oxygen transport. In subjects that received >30 g Hb, pulmonary diffusing capacity increased up to 20 per cent over baseline values. In marked contrast, in patients infused with RL, diffusing capacity decreased 14 per cent below baseline levels. The increase in pulmonary diffusing capacity with HBOC-201 suggested enhanced diffusion of oxygen across the alveolar capillary membrane and was consistent with the observation that hemoglobin in the plasma space facilitates oxygen release to

tissues. Other pulmonary function tests, including spirometry and flow volume loops were unchanged during the study. There were no clinically significant changes in PaO_2, $PaCO_2$, pH or SaO_2 measured either by arterial blood gas analysis or pulse oximetry. Oxygen consumption increased ~21 per cent in the 38-g Hb group compared to an ~10 per cent increase above baseline in the control group. CO_2 production and metabolic energy expenditure were statistically different (slightly increased) from baseline with doses >16 g hemoglobin, but not with an equivalent volume of RL. Arterial oxygen content and calculated oxygen delivery decreased in all groups as expected after phlebotomy and hemodilution, then increased in the HBOC-201 groups (at doses >30 g Hb) but not in the control group. HBOC-201 treated subjects had a small but transient decrease in cardiac index and heart rate and concomitant increase in blood pressure and total peripheral resistance which returned to baseline 8 hours after beginning the infusion. There were no immunologic effects, electrocardiogram changes or laboratory test findings (serum complement, C-reactive protein, Coombs tests, antibodies to erythrocyte antigens) associated with HBOC-201. Side effects observed in both groups included minor transient gastrointestinal events.

In the same model of phlebotomy and volume replacement, Hughes *et al*. (1995a) evaluated the hematologic effects of HBOC-201 (up to 45 g Hb, or 350 ml) administered as a single dose in normal healthy men and women (n = 24). HBOC-201 produced increases in serum iron (100–150 μg/dl) and ferritin that closely paralleled the plasma levels of HBOC-201 with a timing that indicated that the iron from HBOC-201 was normally utilized. Serum erythropoietin levels increased two-fold to six-fold at 24 hours in the HBOC-201 group, without evidence of hypoxemia. Hemoglobin did not appear in the urine of any subjects infused with HBOC-201 and creatinine clearance was unchanged. Hughes *et al*. (1995a) suggested that the ferritin increase in the HBOC-201 group could play an important role in iron replacement in deficiency states.

The effects of HBOC-201 on oxygen transport were studied by Hughes *et al*. (1995b) in a randomized, single-blind, two-way crossover study comparing the effects of HBOC-201 with RL on graded exercise testing in six healthy male subjects. Volunteers underwent a baseline submaximal (65 per cent maximum capacity) bicycle exercise stress test to an aerobic threshold followed the next day by phlebotomy and subsequent hemodilution with RL over 2 hours. The subjects then received either an autologous blood transfusion (~150 g Hb) or an infusion of 45 g Hb of HBOC-201 and were subjected to the exercise test 45 minutes later. A week later, the procedures were repeated, except the volunteers received the opposite treatment.

In this study, exercise tolerance and diffusing capacity were similar to baseline after either autologous blood transfusion (~150 g Hb) or infusion with HBOC-201 (~45 g Hb). Blood pressure was slightly higher (~5 mmHg) during the administration of HBOC-201 with a commensurate increase in total peripheral resistance, generally within the first 4 hours. Cardiac index declined during treatment with HBOC-201 (~0.5 l min/M^2) and pulse rate was about 5–10 beats lower. There were small but transient increases in alanine aminotransferase, aspartate aminotransferase, 5′-nucleotidase, lipase and creatine kinase after treatment with HBOC-201. Adverse events were mild and infrequent. Plasma lactate levels were lower and oxygen uptake and carbon dioxide production were greater after infusion with HBOC-201 than after blood transfusion, suggesting that HBOC-201 may promote improved oxygen metabolism at the cellular level. Furthermore, under the conditions of this study, a 45-g dose of HBOC-201 hemoglobin produced physiologic effects similar to those seen with ~150 g of autologous RBC hemoglobin.

PHASE I/II STUDIES

The Phase I studies, using phlebotomy and volume replacement as a model of 'surgical blood loss', laid the foundation for entry into Phase I/II studies in surgical patients and patients with sickle cell anemia. To evaluate increased doses, an elective surgery study was performed in which the initial dose was comparable to doses tested in the Phase I studies, but with subsequent dosing that was two- to three-fold higher.

Sprung *et al*. (2002) evaluated the tolerability of HBOC-201 in a randomized, single-blind, dose-escalation, RL controlled, multicenter study in 81 patients undergoing non-cardiac surgery who were expected to receive allogeneic blood. Patients were randomized 2 : 1 to receive a single intraoperative dose of either HBOC-201 (n = 55) (0.6 g Hb/kg, 0.9 g Hb/kg, 1.2 g Hb/kg, 1.5 g Hb/kg, 2 g Hb/kg or 2.5 g Hb/kg) or an equivalent volume of RL solution (n = 26) after an estimated 500-ml or more surgical blood loss. A 5-ml test dose of

clinical trial material (either HBOC-201 or RL) was administered prior to anesthesia induction to assess tolerability. The efficacy of treatment with HBOC-201 was evaluated by assessing the number of allogeneic RBC units administered per patient. Safety and tolerability outcomes (evaluated by comparing treatment emergent signs and symptoms) were used to permit progression to the next higher dose level.

The mean number of units of RBC transfused was similar in the two groups. There was no apparent relationship between dose of HBOC-201 and the number of units of allogeneic RBC transfused in a patient. The investigators noted that this was due primarily to study design because only single HBOC-201 doses were investigated; not the consecutive administration of HBOC-201 that was shown in later surgical studies to reduce the use of RBC transfusion.

Intraoperative doses of HBOC-201 (up to a maximum of 245 g) were well tolerated. There were no patient deaths or withdrawals during the study. Adverse events were similar in both treatment groups in type of event and frequency of occurrence; most were not associated with either treatment. Nausea, hypertension, oliguria, skin discoloration after large doses, and rash occurred somewhat more frequently in the HBOC-201 group; fever, hypotension, constipation, insomnia, gastrointestinal disorder and hypomagnesemia occurred somewhat more frequently in the RL group. Administration of HBOC-201 was associated with a dose-dependent increase in methemoglobin concentrations (3.7 per cent $\pm$ 3.2 per cent). Systolic blood pressure was approximately 12 per cent higher in the HBOC-201 treated patients than controls following recovery room discharge; other vital signs were not different. Isolated transient increases in aspartate aminotransferase and/or lipase activities (that returned to normal levels prior to hospital discharge) were observed in a few patients. One serious adverse event (mast cell degranulation syndrome) occurred in a hyperimmune patient with systemic mastocytosis who received only a test dose of HBOC-201; the event was considered of unknown association with HBOC-201. Other serious adverse events were not associated with treatment.

Two additional, controlled, perioperative Phase I/II studies, conducted in Europe, assessed the safety of HBOC-201 in acute normovolemic hemodilution. These low-dose studies (Kasper *et al*., 1996, 1998; Brauer *et al.,* 1998; Standl *et al*., 1998) demonstrated that HBOC-201 was well tolerated during acute normovolemic hemodilution (in patients undergoing liver resection and elective surgery for aortic abdominal aneurysms).

In the patients undergoing preoperative hemodilution prior to elective abdominal aortic surgery, Kasper *et al*. (1996, 1998) investigated the effects of HBOC-201 on hemodynamics and oxygen transport in a single-blind, randomized, single-center, escalating single-dose, hydroxyethylstarch (HES) controlled pilot trial (n = 13) and two subsequent studies (total n = 24). Patients (randomized 1 to 1) received a single infusion of HBOC-201 (doses of 0.4 g Hb/kg to 1.2 g Hb/kg) or an equal volume of hydroxyethyl starch (HES) during hemodilution with RL after anesthesia induction and approximately one hour before surgery. Infusion of HBOC-201 caused an increase in systemic and pulmonary vascular resistance and decrease in cardiac index while maintaining arterial oxygen content at near baseline levels. Base excess, which remained unchanged in the HES treated group, was improved significantly in the HBOC-201 groups 30 minutes after infusion while oxygen consumption was similar in both groups. The improvement in arterial base excess and maintenance of arterial oxygen content in these patients suggest enhanced tissue oxygen transport, despite reduced cardiac output.

Although Kasper *et al*. (1996, 1998) concluded that hemodilution with HBOC-201 (at the doses used in these studies) provided no advantage over hemodilution with HES, they did not directly measure tissue oxygenation. Characteristically, treatment with HBOC-201 results in a decrease in cardiac output associated with small increases in systemic vascular resistance however, this is associated with marked and sustained increases in oxygen extraction and increases in tissue oxygenation as demonstrated by preclinical studies performed by Standl *et al*. (1997). These studies showed a primary effect of an increased oxygen extraction ratio associated with a transient decrease in cardiac index. This physiology, which is supported by data from Page *et al*. (1998), highlights the fact that hemoglobin solutions increase diffusive oxygen transport as well as convective oxygen transport. Therefore, HBOC-201 causes a primary effect on tissue oxygen extraction because of its unique ability to transport high concentrations of oxygen in the plasma that is readily transported to tissues. Autoregulatory mechanisms then result in an appropriate decrease in cardiac output caused by the increased oxygen extraction. The Standl experiments further define this relationship and

show that these changes are associated with normal or above normal tissue oxygen levels as measured by the Eppendorf microelectrode technique. The Kasper studies (1996, 1998) demonstrated that HBOC-201 maintained oxygen through a higher extraction ratio and a lower cardiac output whereas the HES group maintained oxygen consumption by increasing cardiac output without an increase in the oxygen extraction ratio.

Liver resection

Standl *et al*. (1998) investigated the use of HBOC-201 in a randomized, single-blind, single-center, single-dose, HES-controlled study to evaluate the safety and tolerance of HBOC-201 in patients undergoing preoperative hemodilution prior to elective liver resection surgery. Patients were randomized 1 : 1 to receive a single infusion of HBOC-201 (0.4 g/kg, ~1 unit) or HES (equal volume) during hemodilution with RL prior to surgery. There were no differences in heart rate, respiratory rate, temperature, pulse oximetry, blood-gas tensions, or blood chemistry parameters between the two groups throughout the 3-month monitoring period. Patients in the HBOC-201 group had a higher MAP than the control group upon admission to the intensive care unit (95 versus 83 mmHg, respectively; $P = 0.037$) and more pronounced leukocytosis and reticulocytosis during the early postoperative days compared to the HES treated patients. There was a slight increase in methemoglobin with HBOC-201, but the increase remained within a clinically acceptable range. HBOC-201 was not associated with toxic effects nor was it associated with any allergic reactions. In addition, no adverse events were considered by the investigator to be associated with treatment with either HBOC-201 or HES. The authors concluded that HBOC-201 was well tolerated in this patient population and appeared to be safe as a substitute fluid for preoperative hemodilution.

As part of this study in patients undergoing liver resection, Brauer *et al*. (1998) used transcranial doppler sonography (TCD) to measure cerebral blood flow before and after infusion of HBOC-201 or HES. After treatment there was a slight increase (not statistically significant) in TCD mean flow velocity compared with baseline measurements, which was not different between the two treatment groups and was likely due to hemodilution (decreased hematocrit). These data demonstrate that infusion of HBOC-201 did not result in any specific cerebrovascular side effects compared to control.

Sickle cell disease

Due to its unique oxygen transport characteristics (circulation in the plasma, and its effects on oxygen-diffusing capacity), HBOC-201 may be beneficial for managing the vaso-occlusive crises in patients with sickle cell disease (SCD). Gonzalez *et al*. (1997) and Orringer *et al*. (1995) measured local perfusion in sickle cell anemia patients not in crisis in a randomized, single-blind, single-dose, dose-escalation, saline-controlled, single-center study. Patients were randomized 2 : 1 to treatment with HBOC-201 (at 0.2, 0.4 or 0.6 g Hb/kg) or an equal volume of saline. The study monitored treatment-emergent signs and symptoms, and determined the functional capacity of selected muscle groups by grip strength and aerobic selected muscle group evaluations. Pulmonary function tests were performed on patients in the two higher-dose groups.

HBOC-201 was well tolerated at all doses. Systolic and diastolic blood pressures were nearly identical at all time points, and there were only minor changes in hematological and chemical laboratory parameters. One patient receiving 0.2 g Hb/kg of HBOC-201 experienced a painful sickle cell crisis attributed to the 4-hour post-infusion exercise test but this was not considered associated with HBOC-201. There were no significant differences between the two groups in handgrip testing. However, when subjects underwent graded exercise testing, heart rate during aerobic exercise was significantly lower in the HBOC-201 group ($P = 0.0061$) even though exercise intensity was similar in both patient groups. By 48 hours post-infusion, heart rate was similar in both the HBOC-201 and placebo patients. The investigators hypothesized that HBOC-201 may facilitate oxygen delivery and help overcome the rheologic abnormality of sickle erythrocytes in SCD patients.

PHASE II STUDIES

The Phase I and Phase I/II studies included a range of subjects and surgical populations in which the safety of HBOC-201 was assessed. The demographic profile included both men and women, a wide range of ages, as well as low- to

high-volume blood loss. HBOC-201 was administered as single doses, based upon the physiologic observations from the Phase I data. These studies demonstrated the safety of HBOC-201 at doses that would maintain plasma hemoglobin levels greater than 1 g/dl and led to two major surgical trials (Phase II), one in cardiopulmonary bypass patients and one in patients undergoing abdominal aortic reconstruction. While these Phase II studies continued to evaluate safety, specific endpoints related to efficacy and oxygen transport (elimination/reduction of allogeneic RBC transfusions) were also assessed.

Cardiac surgery

In a multicenter, double-blind, variable-dose, red blood cell (RBC) controlled, comparative, parallel-group study (n = 98), Levy *et al*. (2002) investigated treatment with HBOC-201 as an alternative to RBCs in patients requiring transfusion in the immediate post-cardiopulmonary bypass period. In this trial, HBOC-201 treatment began after the surgical procedures had concluded. While patients were in the intensive care unit they were randomized 1 : 1, at the first postoperative transfusion decision, to treatment with RBC or HBOC-201 for up to three transfusion decisions within 72 hours. Patients in the HBOC-201 group received 60 g hemoglobin at the first transfusion decision and 30 g hemoglobin at each of the next two transfusion decisions to give a total dose of 120 g hemoglobin. Patients in the RBC group received 1 unit allogeneic RBC per transfusion decision. Subsequent transfusion requirements for both groups were met with RBC.

Results of the study showed that, in spite of the limited dosing, 34 per cent of the patients treated with HBOC-201 avoided transfusion with allogeneic RBC post-surgery. In addition, HBOC-201 significantly reduced the median number of RBC units administered per patient in the HBOC-201 group (1 unit) compared to the RBC group (2 units) ($P = 0.01$). Even though HBOC-201 treatment does not cause an increase in hematocrit, the hematocrit levels, which were initially much lower in the HBOC-201 group, were similar to the RBC group at discharge and again at follow-up. This effect was attributed to both hemoconcentration as well as enhanced build-back of endogenous RBCs.

The incidence of serious adverse events, changes in vital signs, ECG abnormalities and other safety findings were similar in the two treatment groups. MAP was increased in the HBOC-201 treated patients, but there was no difference in maximum MAP between the two groups. Patients in both groups had increases in oxygen delivery index and oxygen consumption index, relative to pre-treatment values, but cardiac index was lower and oxygen extraction was significantly higher ($P = 0.05$) in the HBOC-201 group. Fourteen patients in the HBOC-201 group experienced non-clinical jaundice (skin discoloration due to HBOC-201 circulation in the plasma phase), and all cases were resolved by discharge. Complication rates were similar in both treatment groups, but one patient in the HBOC-201 group died after a failed intubation attempt (of aspiration pneumonia); this was not considered to be associated with HBOC-201. The study was not powered to find differences in morbidity or mortality.

Abdominal aortic surgery

The second Phase II surgery trial (LaMuraglia *et al.*, 2000) was a multicenter, randomized (2 : 1), single-blind, variable-dose, RBC-controlled, comparative, parallel-group study to evaluate the effect on allogeneic blood use and the safety of HBOC-201 when administered to patients (n = 72) undergoing abdominal aortic reconstruction (AAR). This study allowed administration of HBOC-201 earlier in the treatment of the patients, either during the surgical procedure or any time post-surgery. The trial increased the total dose of HBOC-201 to 150 g hemoglobin, which could be administered in a single dose or in divided doses over a 4-day period dependent on patient need. Patients in the HBOC-201 group received 60 g hemoglobin at the first transfusion decision and 30 g hemoglobin at each of the next three transfusion decisions. Patients in the RBC group received 1 unit of allogeneic RBC at each of the first four transfusion decisions. Subsequent transfusion requirements for both groups were met with RBC. The primary efficacy endpoint was the percentage of patients in the HBOC-201 group who avoided allogeneic RBC use for a 28-day perioperative period. Following treatment with HBOC-201, allogeneic blood transfusions were avoided by 27 per cent (95% CI, 15 per cent to 42 per cent) of patients. In contrast to the results of the cardiopulmonary bypass study, the overall median number of allogeneic RBC units transfused was similar in both groups and likely reflects the greater blood loss in this patient population and

protocol-defined limits on the total maximum dose of HBOC-201 allowed.

The incidence of adverse events was similar for the two treatment groups, although rashes were more common in the HBOC-201-treated patients ($P < 0.1$). The majority (14/17) of patients who experienced a rash were from one study center and required no treatment. Although asymptomatic, the areas of skin where the rash appeared were delineated by a particular adhesive film used to cover the operative field. Adverse reactions that were more common ($P < 0.1$) in the RBC group included postural hypotension, vesiculobullous rash, dyspepsia, ketosis, monocytosis, hypesthesia, confusion and abnormal mentation. Laboratory abnormalities (including creatinine) were similar in both treatment groups except for serum urea nitrogen, which was increased significantly in the HBOC-201 treatment group and thought to be the result of the high protein load. Small increases (approximately 10 mmHg) in mean systolic blood pressure occurred in the HBOC-201-treated patients only after the first treatment. Changes from baseline in mean pulmonary arterial pressure, pulmonary arterial occlusion pressure, cardiac index (CI) and oxygen delivery index were similar in both groups. Furthermore, in the HBOC-201 group, base excess was significantly better, suggesting more efficient oxygen release from HBOC-201 than from stored RBCs. Two patients (8 per cent) in the RBC group and three patients (6 per cent) in the HBOC-201 groups died ($P = 1.0$).

PHASE III STUDY

Since not only was HBOC-201 well tolerated in the cardiopulmonary bypass and abdominal aortic reconstruction patient populations, but also efficacy was demonstrated by the elimination of allogeneic RBC transfusions in 34 per cent and 27 per cent, respectively, of patients who received HBOC-201, clinical evaluation of the product continued. In the progression of clinical trials, the same logistic and design features of the AAR trial were extended to the Phase III surgery trial in patients undergoing elective orthopedic surgery with an increase in total hemoglobin dose to 300 g (10 units).

Orthopedic surgery

A multicenter, multinational, randomized, single-blind, RBC-controlled Phase III study (Jahr, 2002) was performed to evaluate the safety and efficacy of HBOC-201 in patients undergoing elective orthopedic surgery (n = 688). Patients were randomized 1 : 1 to receive either HBOC-201 or RBC at the time of the first perioperative allogeneic RBC transfusion decision. The randomization was stratified by type of surgery, patients undergoing procedures involving the back (e.g. spinal fusion) and patients undergoing procedures that did not involve the back (e.g. hip arthroplasty). Subjects participating in the study were at least 18 years of age (no upper age limit), had not received erythropoietin or undergone PAD, were to undergo surgery within 24 hours, were undergoing (or had undergone) non-emergency orthopedic surgery prior to enrollment and randomization, and were expected to require at least 2 units of RBC transfusion. Efficacy was determined by the proportion of patients in the HBOC-201 treatment group who did not receive any transfusions of allogeneic RBC during the study following treatment with HBOC-201. Safety was determined by assessing the frequency and severity of treatment emergent adverse events, as well as by evaluating physical condition, vital signs and clinical laboratory tests.

In this study, 59.4 per cent of the patients treated with HBOC-201 required no RBC transfusion from randomization to follow-up. Furthermore, in the HBOC-201-treated group, 96.3 per cent of the patients avoided transfusion with RBC on the first postoperative day and 70.3 per cent avoided transfusion with RBC up to treatment day 7. As expected, treatment with HBOC-201 resulted in decreased initial hematocrit levels, but the hematocrit levels were similar for the two groups by discharge and again at follow-up. Adverse events in the HBOC-201 group that showed an absolute difference of greater than 5 per cent compared to the RBC group were anemia, tachycardia, abdominal pain, diarrhea, dysphagia, nausea, vomiting, pyrexia, jaundice, lipase increases, oliguria and hypertension. The authors concluded that these data support the use of HBOC-201 as an oxygen-carrying solution in orthopedic patients.

OTHER POPULATIONS AND CASE STUDIES

Abdominal aneurysm

Sprung *et al*. (2001) described the use of HBOC-201 in a 62-year-old man (a participant in a Phase II clinical trial discussed above) admitted for a 7-cm infrarenal abdominal aneurysm repair. Upon resection of the aneurysmatic aorta, there was an

acute 600-ml blood loss (collected in the cell saver) with an estimated total blood loss for the surgery of 8 l (~1.4 times the blood volume). The patient was infused continuously with HBOC-201 (1.2 g/kg at a rate of 59.3 g (2 units, 500 ml) per hour), along with a crystalloid infusion. In total, the patient received 2.5 l of salvaged blood, 2 units of autologous fresh-frozen plasma, 1 l HES, 11.5 l of crystalloids and 0.89 l HBOC-201. After infusion of HBOC-201, the patient's intra-operative hemodynamic course was stable (normal blood pressure and cardiac output). By postoperative day 5 the hemoglobin level was 8.3 g/dl, with a hematocrit of 25 per cent, and there were no clinical signs of anemia. Throughout 2 weeks of hospitalization, the patient's vital signs were normal and he required no banked RBCs. The authors concluded that the remarkable hemodynamic stability of this patient was a result of the enhanced oxygen delivery and volume stabilization provided by HBOC-201 following administration in an early stage of hemorrhage.

Intraoperative myocardial ischemia

Preclinical studies (Horn *et al*., 1997; Standl *et al*., 1999; Standl, 2001) have demonstrated that HBOC-201 results in increased oxygen diffusion and tissue perfusion when restricted vessel size does not allow RBC flow. Similar effects were seen by Niquille *et al*. (2000) in a 64-year-old man (enrolled in an HBOC-201 clinical trial) who was hospitalized for occlusion of the right lower limb of an aortobifemoral graft. During aortic cross-clamping, the patient developed progressive systolic and diastolic hypotension (to 80/50 mmHg) and a new 2-mm ST-T segment depression on standard leads II and V5. Dobutamine and nitroglycerin administration increased blood pressure and heart rate with no effect on the ST-segment depression. Administration of 60 g (500 ml) of HBOC-201 resulted in a slight increase in systemic arterial blood pressure and a decrease in heart rate with rapid normalization of the ST-T segment. Postoperatively, recurrent ST-T changes and tachycardia resolved after a second infusion of 30 g of HBOC-201. The only adverse effects seen in the patient were transient skin discoloration and increase in aspartase aminotransferase without clinical or ultrasound evidence of hepatic disease. Niquille *et al*. (2000) attributed the hemodynamic stability and normalization of ECG changes to improved tissue oxygenation due to treatment with HBOC-201.

Autoimmune hemolytic anemia

HBOC-201 does not contain RBC membrane antigens, suggesting that its use as a treatment for autoimmune hemolytic anemia may be beneficial. Mullon *et al*. (2000) used HBOC-201 under compassionate use for the treatment of a 21-year-old woman with idiopathic thrombocytopenic purpura. She became acutely anemic and her antibodies reacted with erythrocytes from all available donors, causing profound hemolysis with fever, nausea and back pain. A total of 11 units of HBOC-201 (330 g) were administrated over a 7-day period until resolution of the hemolytic anemia, for clinical signs of end-organ ischemia (acidosis, serum lactic acid of 2.2 mmol/l) (5 units), hemodynamic instability (MAP of 40 mmHg) during septic shock (3 units), and a total hemoglobin level less than 4 g/dl (3 units). The average total hemoglobin level during HBOC-201 therapy was 5.5 g/dl with a hematocrit of 9.5 per cent, but there were instances when the hematocrit was so low that most of the oxygen-carrying capacity in the circulation was due to HBOC-201. Hemodynamic monitoring revealed normal MAP during therapy (average of 93.6 mmHg), slight increases in pulmonary arterial systolic and diastolic pressures, and a septic shock-associated decrease in cardiac index which improved as MAP normalized and the shock resolved. The investigators concluded that HBOC-201 was lifesaving in this patient with refractory autoimmune hemolytic anemia.

HBOC-201 interference with clinical laboratory analyses

Hemolysis has long been recognized to interfere with laboratory analyses through the release of red cell contents (electrolytes and proteins), direct spectral interference from hemoglobin, and interference of hemoglobin or its derivatives with chemical reactions. The presence of HBOC-201 in serum or plasma samples also can cause interferences; however, the interferences are not identical to that observed from hemolysis (Callas *et al*., 1997; Moreira *et al*., 1997). The degree of interference from HBOC-201 depends upon the instrument, analyte, reagent, methodology, HBOC-201 concentration and analyte-to-reagent ratio. Interferences may lead to increases or decreases in laboratory test results. Thus, there is no straightforward means of correcting an analyte result for HBOC-201 interference.

Several published studies (Ma *et al*., 1997; Moreira *et al*., 1997; Wolthuis *et al*., 1999) have examined the effects of HBOC-201 on routine clinical laboratory tests; however, since instruments and methodologies change as techniques are developed and advancements are made, these data should be verified with current instrumentation. Wolthuis *et al*. (1999) found that HBOC-201 did not interfere with routine hemocytometry, hemostasis analysis or detection of RBC agglutination. HBOC-201 was found to affect direct bilirubin, creatine kinase MB-fraction, creatine kinase, gamma-glutamyl transferase, magnesium and uric acid analysis.

A second study examined the concentration dependence of HBOC-201 interference on 22 chemistry tests performed with Ektachem/Vitros and Hitachi analyzers (Moreira *et al*., 1997). Assays that were not color-dependent (sodium, potassium, chloride) were not affected by the presence of HBOC-201 in the specimens. Measurement of aspartate aminotransferase, calcium, urea nitrogen, creatine kinase and glucose also were not affected. However, the analyzers tested could not accurately measure albumin, bilirubin, alkaline phosphatase and lactate dehydrogenase in the presence of HBOC-201. The investigators found that Ektachem analyzers showed superior performance for measurements of creatine kinase, lipase, magnesium and uric acid, while the Hitachi analyzers were better for the analysis of alanine aminotransferase, amylase and total protein (Table 37.3).

In general, analytes measured in the chemistry laboratory showed the most interference with HBOC-201. Although interference results vary depending upon reagent, methodology and equipment, a few chemistry analytes nearly always show some degree of interference with HBOC-201. In particular, alanine aminotransferase, amylase, bilirubin, gamma-glutamyl transferase, lipase, phosphorus, total protein and uric acid assays often are not available immediately after HBOC-201 treatment.

Jahr *et al*. (2002) found that mechanical detection methods (fibrometer, STA, CS-190) and MDA-180 methods were less affected by increasing levels of HBOC-201 than were optical detection devices for all coagulation test parameters. Since pulse oximetry (SpO_2) is frequently used to evaluate percentage saturation of hemoglobin, Hughes *et al*. (1996b) investigated whether this test could accurately measure oxygenation in patients receiving HBOC-201. They found similar correlations between the values obtained from blood gas analyzers and SpO_2 in the HBOC-201 group and the control group receiving RL in 271 pairs of tests. Subsequently, Sprung *et al*. (2002) reported that neither occasional skin discoloration nor discoloration of serum associated with HBOC-201 compromised SpO_2 monitoring. These findings demonstrate that oxygen saturation can be accurately measured in the presence of low doses of HBOC-201 with a number of standard measurement techniques. Oxygen saturation measurements will likely be affected by large doses of HBOC-201 due to the fact that its oxygen dissociation curve differs from that of red blood cells. More specifically, the HBOC-201 oxygen dissociation curve (see Chapter 36, Figure 36.1) shows a higher $P50$ than native hemoglobin (40 ± 6 mmHg versus 27 ± 1 mm Hg) as well as a lower maximal oxygen saturation. The lower maximal oxygen saturation is likely due to the fact that polymerization of the hemoglobin molecules results in a non-cooperative configuration; thus polymerized hemoglobin is not as completely saturated as red blood cell hemoglobin at normal arterial PO_2.

Pulse oximeter readings accurately reflect changes in a patient's combined RBC and HBOC-201 hemoglobin saturation. This is because the absorbance spectra for HBOC-201 is nearly identical to human hemoglobin and the pulse oximeter measures at absorbencies of 660 nm and 940 nm. However, a misinterpretation of the pulse oximeter readings can occur. The clinician may observe a slight decrease in the pulse oximeter reading (e.g. 2–4 per cent) and assume the patient is becoming hypoxemic when in fact the oxygen tension of the patient has not changed as measured by blood gas analysis. The decrease in the pulse oximeter reading (hemoglobin saturation) is due to the right shifting of the HBOC-201 oxygen dissociation curve owing to the presence of plasma hemoglobin.

While clinical chemistry analyses show the most interference, every laboratory instrument that may be critical for patient care – including blood typing and crossmatching, coagulation and point-of-care instruments – should also be assessed for interferences.

SUMMARY

The efficacy of HBOC-201 has been demonstrated in numerous preclinical studies, Phase I safety

Table 37.3 Average per cent assay variation with Hemopure®, with maximum Hemopure concentrations for accurate Hitachi and Ektachem/Vitros chemistry analysis (source, Moreira, 1997)

Analyte conc.	Hitachi 747			Ektachem 700		
	10 g/l	30 g/l	50 g/l	10 g/l	30 g/l	50 g/l
ALT, 116 U/l	0	+5	−25	−40	−78	UTQ
AST, 123 U/l	+3	+9	−7	+3	+11	+18
Albumin, 2.0 g/l	+73	+202	+308	+72	UTQ	UTQ
Alkaline phosphatase, 60 U/l	UTQ	UTQ	UTQ	UTQ	UTQ	UTQ
Amylase, 55 U/l	ND	ND	ND	+42	+104	+155
Bilirubin, 60 μmol/l	−22	+48	+134	+52	+159	+317
Urea nitrogen, 17 mmol/l	+2	+3	+3	+7	+8	+7
Calcium, 1.7 mmol/l	+2	+8	+9	+3	+6	+7
Chloride, 108 mmol/l	−3	−3	−3	+1	+1	0
Cholesterol, 3.3 mmol/l	+16	+36	+73	−6	−22	−30
Creatine kinase, 366 U/l	−16	−8	−12	−11	−6	−10
Creatinine, 398 μmol/l	−15	−51	−84	−4	−14	−17
GGT, 122 U/l	+26	UTQ	UTQ	+27	UTQ	UTQ
Glucose, 0.9 mmol/l	+2	+2	+1	−2	−1	+2
Lactate dehydrogenase, 1031 U/l	−30	−56	−44	UTQ	UTQ	UTQ
Lipase, 151 U/l	ND	ND	ND	−1	−2	−5
Magnesium, 1.1 mmol/l	+12	+17	+23	+5	+8	+14
Phosphorus, 1.49 mmol/l	−1	0	−1	+4	+13	+23
Potassium, 4.0 mmol/l	0	−1	−3	+3	+3	0
Sodium, 145 mmol/l	−1	−2	−3	+2	+2	+2
Total protein, 40–130 g/l	−1	−9	−16	+25	+55	UTQ
Uric acid	+10	+39	+99	−3	−7	−9

	Maximum Hemopure® without interference, g/l				
Assay	Hitachi 717	Hitachi 747	Hitachi 911	Ektachem 950	Ektachem 700
ALT	40	39	25	4	0
AST	50	50	50	50	50
Albumin	0	0	0	0	0
Alkaline phosphatase	0	0	0	0	0
Amylase	0	7	20	0	0
Bilirubin	0	0	0	0	0
Urea nitrogen	50	50	50	50	50
Calcium	50	50	50	50	50
Chloride	50	50	50	50	50
Cholesterol	10	12	9	25	20
Creatine kinase	50	50	50	50	50
Creatinine	4	8	6	30	25
GGT	0	0	4	0	0
Glucose	50	50	50	50	50
Lactate dehydrogenase	0	0	0	0	0
Lipase	20	ND	20	50	30
Magnesium	0	0	5	45	40
Phosphorus	13	13	50	14	20
Potassium	50	50	50	50	50
Sodium	50	50	50	50	50
Total protein	7	25	10	0	0
Uric acid	0	0	0	30	45

GGT, γ-glutamyltransferase; UTQ, unable to quantify; ND, not done on this instrument.

and exercise tolerance studies in volunteers, and in RBC-controlled clinical trials in patients undergoing surgery. In the Phase I studies, a plasma hemoglobin concentration of ⩾0.6 g/dl was associated with a greater than 20 per cent elevation of arterial oxygen content, a 10 to 15 per cent enhancement of pulmonary diffusing capacity over baseline, and improved exercise tolerance. These outcomes can be linked to a variety of physiological effects on oxygen delivery/utilization and hemodynamics. In the RBC-controlled clinical trials, clinically significant proportions of patients administered HBOC-201 were able to eliminate the need for allogeneic RBC transfusions. Elimination was 34 per cent, 27 per cent and 59 per cent for the cardiopulmonary bypass, abdominal aortic reconstruction and orthopedic surgery trials, respectively. In addition, in two of these studies the median number of allogeneic RBC units administered to the HBOC-201 group was reduced by 1 unit when compared to the RBC group. These studies, with increasing doses over extended periods of time, demonstrate that HBOC-201 may provide a temporary oxygen transport bridge until endogenous RBC mass can restore adequate oxygen-carrying capacity, especially when RBCs are not immediately available but surgery is urgently required.

HBOC-201 was well tolerated in a range of doses and regimens, and in a variety of clinical, particularly perioperative, settings.

Continuing research supports expanding the use of HBOC-201 to the treatment and management of trauma and ischemia. Severely injured patients in particular may have an emergent requirement for early blood transfusions, especially in the pre-hospital setting where blood is rarely available and the oxygen delivery by HBOC-201 can be lifesaving. In addition, HBOC-201 may be beneficial in the management of ischemia, where tissue perfusion by RBCs may be limited by blood vessel constraints.

EDITOR'S SUMMARY

Phase I studies in normal volunteers demonstrated a disappearance half-time from plasma of about 20 hours, and the data fit a single compartment model. The product was well tolerated, although there were 'minor' incidents of elevated blood pressure and gastrointestinal complaints as well as some elevations of liver enzymes and lipase.

An interesting study of hemodynamics in normal volunteers given approximately 350 ml of HBOC-201 reported increased blood pressure, and decreased heart rate and cardiac output, indicating increased peripheral resistance. These subjects, however, showed significantly increased pulmonary diffusing capacity compared to volunteers who received Ringer's lactate.

Based on these early safety studies, Phase II trials were performed in several settings including elective orthopedic and cardiac surgery. One European study reported increased pulmonary artery pressures and resistances.

A large Phase III international trial in orthopedic surgery patients then followed, which showed significantly reduced use of allogeneic blood transfusion. However, the company also reported significantly increased incidence of certain adverse events, including anemia, tachycardia, abdominal pain, diarrhea, dysphagia, nausea, vomiting, pyrexia, jaundice, lipase increases, oliguria and hypertension.

The development of HBOC-201 is a good illustration of the changing landscape of 'blood substitute' development. Two decades ago, the benefit of reduced allogeneic blood transfusion would have outbalanced relatively minor adverse events, not leading to loss of life or serious morbidity. However, today blood transfusions are so safe that a comparative study to show superior safety of a blood substitute compared to blood would require so many patients as to be a practical impossibility. Therefore developers of products, especially where there are even minimal side effects, must look to clinical applications where morbidity and mortality can be reduced by the product and these reductions can be shown in clinical trials of reasonable power and, therefore, cost.

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Chapter 38

Polyhemoglobin–Enzymes as New-Generation Blood Substitutes and Oxygen Therapeutics

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INTRODUCTION

The first polyhemoglobin was prepared by crosslinking stroma-free hemoglobin using a bifunctional agent, sebacyl chloride (Chang, 1964; Figure 38.1a). Since stroma-free hemoglobin contained red blood cell enzymes, this crosslinking also resulted in a polyhemoglobin–enzyme complex. The first use of another bifunctional agent, glutaraldehyde, was to crosslink stroma-free hemoglobin into polyhemoglobin, but with additional catalase added to increase the catalase activity (Chang, 1971; Figure 38.1b). Catalase crosslinked into the polyhemoglobin–catalase complex this way is much more stable than the enzyme in free solution. Thus, the catalase activity of the polyhemoglobin–catalase remained at 98 per cent after 43 days when stored at 4°C (Chang, 1971). On the other hand, the activity of the free catalase in solution fell to 10 per cent in 35 days at 4°C. When stored at the body temperature of 37°C, polyhemoglobin–catalase retained 50 per cent of its catalase activity after 7 days, whereas the activity of the free enzyme fell rapidly to 50 per cent after 1 day. There was not much initial interest on the use of the basic principle of the use of these bifunctional agents to intermolecularly crosslink hemoglobin to form polyhemoglobin or polyhemoglobin-enzyme complexes. Lately, the successful independent development of glutaraldehyde-crosslinked polyhemoglobin for clinical uses (Gould *et al.*, 2002; Sprung *et al.*, 2002) has reinitiated interest in this basic principle of intermolecular crosslinking of hemoglobin to form polyhemoglobin or polyhemoglobin–enzyme complexes. Polyhemoglobin–enzyme complexes are being actively developed as new generation blood substitutes and oxygen therapeutics (Chang, 2003). This chapter will concentrate on (1) polyhemoglobin–catalase–superoxide dismutase and (2) polyhemoglobin–tyrosinase.

POLYHEMOGLOBIN–CATALASE–SUPEROXIDE DISMUTASE

Polyhemoglobin is being used in surgery in the last stages of clinical trials in North America (Gould *et al.*, 2002; Sprung *et al.*, 2002) and in routine clinical uses in South Africa and Russia. Large volumes of up to 10 l (20 units) have been infused into trauma surgery patients (Gould *et al.*, 2002), and polyhemoglobin promises to play an important role in perioperative situations. Interest in using this for other conditions, including severe sustained hemorrhagic shock and

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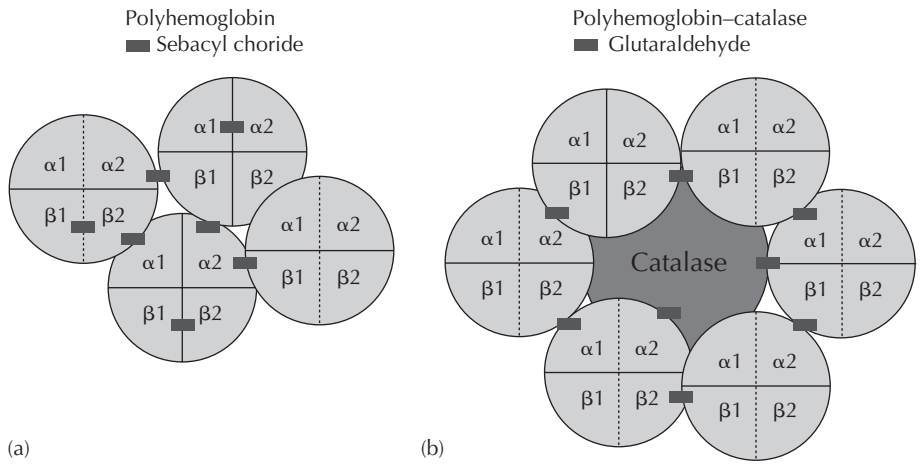

Figure 38.1 (a) First polyhemoglobin formed by intermolecular crosslinking of stroma-free hemoglobin using a bifunctional agent, sebacyl chloride. (Chang, 1964). Since stroma-free hemoglobin contains red blood cell enzymes, the enzymes are also crosslinked into the polyhemoglobin. (b) First use of another bifunctional agent, glutaraldehyde, to crosslink hemoglobin to form polyhemoglobin (Chang, 1971). In addition to red blood cell enzymes present in the stroma-free hemoglobin, additional catalase was added to form a polyhemoglobin–catalase complex.

stroke, raises the question of oxygen radicals (Chang, 1997, 2003, 2004; Winslow, 2003; Alayash, 2004). Red blood cells contain catalase, superoxide dismutase and other enzymes, but in conditions of sustained and severe ischemia even this is not enough. Furthermore, in order to prevent contaminants some polyhemoglobins are prepared from ultrapure hemoglobin devoid of any red blood cell enzymes. We have therefore initiated an ongoing research program in this center to study this (Chang, 2003).

A polyhemoglobin–catalase–superoxide dismutase complex with more catalase and superoxide dismutase than normally present in red blood cells was prepared with each gram of hemoglobin crosslinked with 3000 U of superoxide dismutase and 300 000 U of catalase (Figure 38.2; D'Agnillo and Chang, 1998a). The ratio of hemoglobin to SOD and catalase (in mg/ml) is 1 : 0.009 : 0.0045, thus there is little change in the amount of the oxygen-carrying component, hemoglobin, in this complex when compared to polyhemoglobin. This ratio can be varied over a wide range. We express the ratios of hemoglobin, superoxide dismutase and catalase as mg/ml (1 : 0.009 : 0.0045) instead of molar ratio. This is because the enzyme preparations are not 100 per cent pure, and some have higher specific activities than others. Thus, if we use the enzyme units to calculate the number of enzyme molecules then we are not taking into consideration other potential contaminating proteins. By using mg/ml, we are taking into consideration all sources of protein in the enzyme preparation.

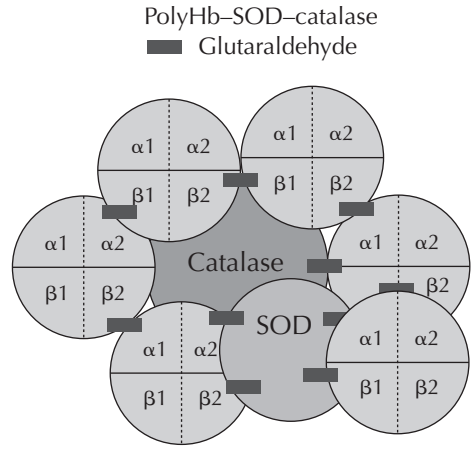

Figure 38.2 Polyhemoglobin crosslinked with superoxide dismutase (SOD) and catalase (CAT) to form polyhemoglobin–catalase–superoxide dismutase (PolyHb–SOD–CAT) (D'Agnillo and Chang, 1997).

We have carried out detailed analysis on methemoglobin formation (Quebec and Chang, 1995). During the cross-linking process, there is negligible formation of methemoglobin when

cross-linking hemoglobin with superoxide dismutase and catalase (Quebec and Chang, 1995). When cross-linking is carried out with hemoglobin alone, there is marked formation of methemoglobin during the preparative procedure. PolyHb also contains higher starting levels of methemoglobin compared to PolyHb–SOD–catalase. Furthermore, during storage methemoglobin levels in the PolyHb solution increase at a greater rate than PolyHb–SOD–catalase. This shows that SOD–catalase may also provide oxidative protection during the preparation and storage of modified hemoglobin solutions (Quebec and Chang, 1995).

Hydrogen peroxide was added to PolyHb (10 mM) or PolyHb–SOD–catalase (10 mM), and the absorbance spectra (450–700 nm) were recorded over time (D'Agnillo and Chang, 1998a). Absorbance spectral recordings were used to monitor the reactions of oxygen free radicals with the hemoglobin components of the crosslinked solutions. Following incubation with equimolar H_2O_2 (10 mM), the spectral changes of PolyHb reflect the oxidation of ferrous (Fe^{2+})-heme producing ferric (Fe^{3+})-heme. With excess H_2O_2, the heme moieties of PolyHb were rapidly degraded. The absorbance spectra of PolyHb–SOD–catalase were minimally affected, indicating these reactions are minimized due to the elimination of H_2O_2. Similar results were recorded following oxidative challenge with exogenous O_2 via xanthine/xanthine oxidase.

We carried out *in vitro* studies to test this based on the reduction of cytochrome c by superoxide with higher concentrations of superoxide shown as higher rates of reduction (D'Agnillo and Chang, 1998b). Each reaction mixture contains either PolyHb or PolyHb–SOD–catalase. Addition of xanthine oxidase starts the reaction at 22°C. The rate of cytochrome c reduction was monitored at 550 nm. The initial rates of cytochrome c reduction were 0.56 nmoles cytochrome c per minute for PolyHb–SOD–catalase compared to 2.13 nmoles cytochrome c per minute for PolyHb, showing fourfold higher remaining superoxide for PolyHb. This shows that PolyHb–SOD–catalase can effectively remove enzymatically-generated superoxide. We found that the superoxide scavenging activity of the polymerized solution increases with increase in SOD concentration used during crosslinking.

Reaction volumes (3 ml) containing the horseradish peroxidase/4-aminoantipyrine/phenol reagent solution (1.2 ml), PolyHb or PolyHb–SOD–catalase (5 μM), water and hydrogen peroxide were prepared. After allowing the mixture to stand for 3 minutes at 22°C, the absorbance at 505 nm was recorded. The results show that, unlike PolyHb, PolyHb–SOD–catalase was effective in scavenging hydrogen peroxide (D'Agnillo and Chang, 1998a, 1998b). Thus, in reaction mixtures containing PolyHb–SOD–catalase, only about 20 per cent of the added H_2O_2 was recoverable at each concentration studied, whereas more than 95 per cent was detectable in PolyHb mixtures. With increasing H_2O_2 concentrations, reactions between PolyHb itself and H_2O_2 became more evident. It was also observed that the H_2O_2 scavenging activity of PolyHb–SOD–catalase increases with the catalase concentration used during crosslinking.

The first step was to incubate PolyHb (15 mM) or PolyHb–SOD–catalase (15 mM) in hydrogen peroxide (total volume; 0.5 ml) for 60 minutes at 37°C. The amount of iron released was calculated by measuring the absorbance of an iron standard (500 μg/dl) (0.5 ml), treated as described above, against blank (0.5 ml H_2O). ($A_{unknown}$/$A_{standard}$* 500). With the addition of 500 mM of H_2O_2, 37 per cent of the total iron in PolyHb was released. For PolyHb–SOD–catalase, less than 1 per cent was released (D'Agnillo and Chang, 1998a, 1998b).

In severe sustained hemorrhagic shock, the intestine is particularly sensitive to ischemia-reperfusion injuries. The intestine has a rich source of xanthine dehydrogenase/oxidase, and it is known to be more vulnerable to ischemia-reperfusion injury. We therefore used this model as a very sensitive model for testing for such injury (Razack *et al.*, 1997).

We studied the effects of PolyHb and PolyHb–SOD–catalase on the production of oxygen radicals using a rat model of intestinal ischemia-reperfusion. After 90 minutes of ischemia, PolyHb or PolyHb–SOD–catalase (5 g/dl) containing 5 mM of 4-hydroxybenzoate was perfused at 0.8 ml/min for 10 minutes without recirculation. Portal vein effluent samples were collected and centrifuged, and the centrifuged perfusate was stored at −8°C for later analysis by high performance liquid chromatography. Hydroxyl radical generation was assessed by an indirect assay based on the hydroxylation of 4-hydroxybenzoate into 3,4 dihydroxybenzoate (3,4 DHBA; Das *et al.*, 1991). Our results show that a significantly higher 3,4 DHBA production occurs during PolyHb perfusion as compared to PolyHb–SOD–catalase (Razack *et al.*, 1997; Figure 38.3).

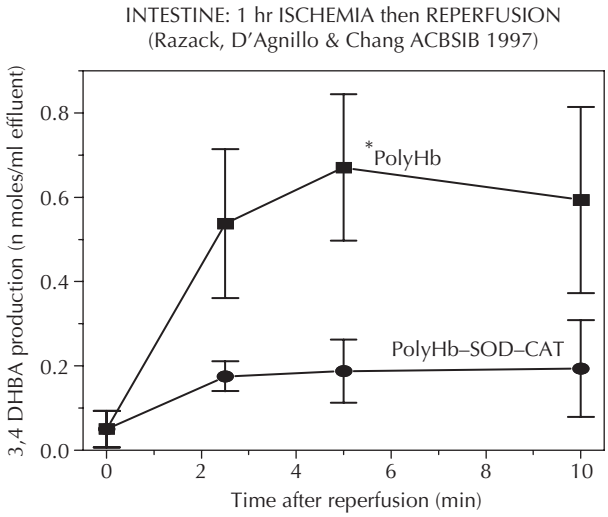

Figure 38.3 Rat model of intestinal ischemia–reperfusion. After 90 minutes of ischemia, reperfusion was started using either polyhemoglobin or polyhemoglobin–catalase–superoxide. Hydroxyl radical generation was assessed by an indirect assay based on the hydroxylation of 4-hydroxybenzoate into 3,4 dihydroxybenzoate (3,4 DHBA) (From Razak *et al*., 1997, with permission).

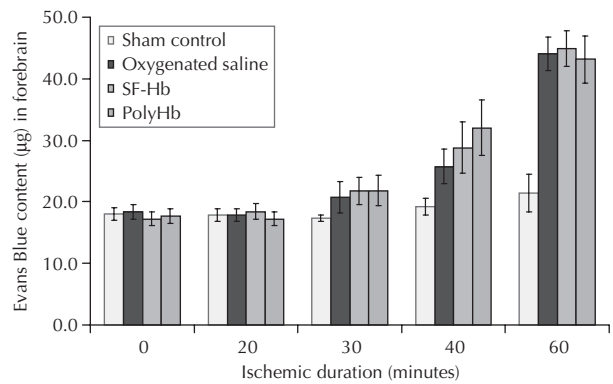

Figure 38.4 Effects of duration of cerebral ischemia on blood–brain barrier measured by extravasations of Evans blue into the brain. Animals were subjected to either 0, 20, 30, 40 or 60 minutes of ischemia before reperfusion using oxygen-carrying solutions (oxygenated saline, stroma-free Hb (SFH) and PolyHb). The maximal effect was observed after 60 minutes of ischemia before reperfusion (from Powanda and Chang, 2002, with permission).

Limbs are known to be much less sensitive to ischemia reperfusion injury. We therefore used this to test the other extreme of sensitivity to ischemia-reperfusion injuries (D'Agnillo and Chang, 1997). We followed the same schedule as for the intestine described above. After 90 minutes of ischemia, reperfusion resulted in minimal production of oxygen radicals as measured by the hydroxylation product, 3,4 dihydroxybenzoate (3,4 DHBA) described above. Nevertheless, this was significantly reduced when we used PolyHb–SOD– catalase instead of PolyHb for the reperfusion (D'Agnillo and Chang, 1997).

Polyhemoglobin in solution, due to its small molecular size as compared to a red blood cell, is able to bypass points of arterial constriction caused by either embolism or thrombosis and deliver the required oxygen supply to the ischemic tissue. Thus it may have a role in stroke or myocardial infarction. Furthermore, since PolyHb has no blood group antigens and can be stored for much longer time than donor blood even at room temperature, there is the potential for using this on site for hemorrhagic shock. However, in all these cases if the ischemia is prolonged before reperfusion there could be ischemia reperfusion injuries. Smith *et al*. (1984) have provided a combined model of hemorrhagic shock and bilateral carotid artery occlusion transient global ischemia-reperfusion rat model. Since the brain is particularly sensitive to ischemia-reperfusion injuries, we used this rat model for the following studies (Powanda and Chang, 2002).

Male Sprague-Dawley rats (235–255 g) were anesthetized with an intraperitoneal injection of pentobartbital (Somnotol™, 65 mg/kg). Blood was withdrawn from the left femoral-artery cannula to maintain the mean blood pressure at 30–35 mmHg. Both common carotid arteries were then occluded with microvascular clips to cut their circulation to the brain.

First, we carried out studies to analyze the relationship between the duration of ischemia before reperfusion and the severity of ischemia-reperfusion injuries using ischemia times of 0 (control), 20, 30, 40 and 60 minutes. After 1 hour of reperfusion using oxygen carriers containing no enzymes, the effect of the duration of ischemia before reperfusion on the blood–brain barrier was measured using Evans blue extravasation. This measures the leakiness of the normally tight intercellular endothelial junctions of the brain capillaries. Since 1 hour of ischemia produced the most significant effect, this was chosen for the following ischemia-reperfusion studies (Figure 38.4).

Ischemia was terminated at 1 hour by removing the bilateral carotid artery occlusions and restoring the blood pressure by replacing the lost blood

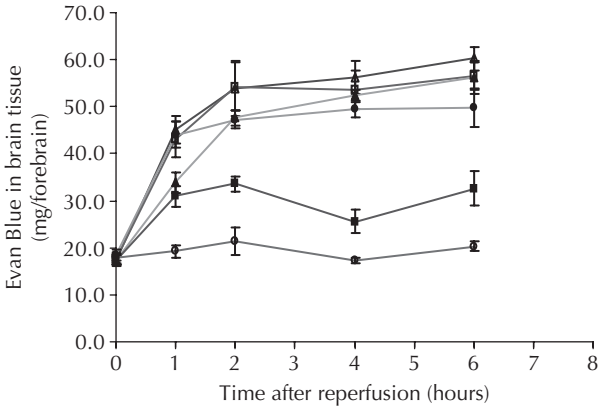

Figure 38.5 Effects of PolyHb–SOD–CAT on blood–brain barrier. PolyHb–SOD–CAT significantly attenuated the severity of blood–brain barrier disruption as compared to reperfusion with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb. $P < 0.01$. (From Powanda and Chang, 2002, with permission.) See color plate 16.

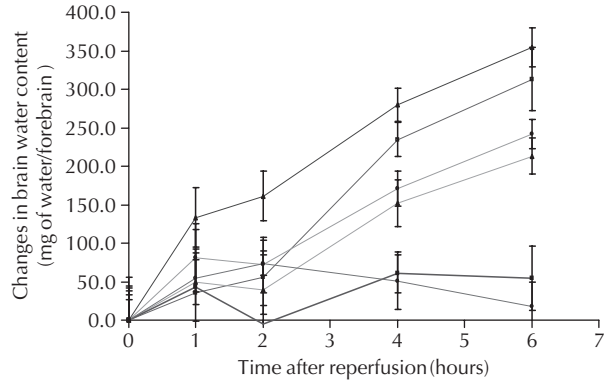

Figure 38.6 Brain edema: changes in brain water content. The changes in brain water content of PolyHb–SOD–CAT treated animals were not significantly different from that of the sham control. The increase in water content when reperfused with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb was significantly different from that of the sham control and PolyHb–SOD–CAT group by the fourth hour and increased there on with time. $P < 0.01$. (From Powanda and Chang, 2002, with permission.) See color plate 17.

using one of the following solutions: oxygenated saline, PolyBvHb (Biopure), ultrapure Hb containing solutions of superoxide dismutase and catalase, PolyHb, or PolyHb–SOD–catalase. Animals from the sham control group were subjected to the same surgical procedure as described above, with the exception that ischemia was not induced.

Disruption of the blood–brain barrier was measured using influx of Evans blue into cerebral tissues. Cerebral tissue Evans blue levels of rats receiving Poly–SOD–catalase and sham control rats remain unchanged when followed for 2, 4 and 6 hours (Powanda and Chang, 2002; Figure 38.5). By 2 hours after reperfusion there were already significant increases of cerebral tissue Evans blue in the groups receiving oxygenated saline ($47 \pm 2\,\mu g$), PolyHb ($54 \pm 4\,\mu g$), stroma-free Hb ($54 \pm 6\,\mu g$), and stroma-free Hb containing solutions of SOD and catalase ($48 \pm 2\,\mu g$ and $56 \pm 4\,\mu g$ by the 6th hour) (Figure 38.5).

Cerebral edema was measured as changes in cerebral water content. There was no significant difference between the PolyHb–SOD–catalase and sham control groups when followed for 2, 4 and 6 hours (Powanda and Chang, 2002; Figure 38.6). On the other hand, significant increases in cerebral water content were observed after 4 hours for PolyHb (234.8 ± 21.9 mg), Hb–SOD–catalase (152.3 ± 30.2 mg), oxygenated saline (171.3 ± 23.0 mg) and SFH (279.8 ± 21.8 mg). Except for PolyHb–SOD–catalase, all the other solutions, like oxygenated saline, SFH and PolyHb, resulted in cerebral edema. It is therefore likely that cerebral edema is related to ischemia-reperfusion injury.

POLYHEMOGLOBIN–TYROSINASE

Melanoma now represents the fifth most common cancer in North America, and its incidence has increased dramatically in the past decade. Surgical incision is effective in early, localized lesions. However, once it spreads there is little that can be done despite different treatment regimes. Tumors are well vascularized but have abnormal microcirculations, resulting in underperfusion by red blood cells and therefore lower tissue oxygen tension. As polyhemoglobin is in solution, it can perfuse the abnormal microcirculation of tumors more effectively than red blood cells to supply more oxygen, at least in theory. Thus polyhemoglobin has been used as an adjunct to increase the efficiency of chemotherapy therapy (Pearce and Gawryl, 1998), and conjugated hemoglobin has been used to increase the efficiency of radiation therapy (Shorr *et al.*, 1996). Meadow's group

(Uhlenkott *et al.*, 1996) has shown that lowering of the tyrosine level can inhibit the growth of melanoma in cell culture and in mice bearing B16BL6 melanoma. However, human subjects cannot tolerate the tyrosine-restricted diets due to nausea, vomiting and severe body weight loss. We therefore prepared a novel soluble polyhemoglobin–tyrosinase complex (Figure 38.7; Yu and Chang, 2004a, 2004b). This has the dual function of supplying the oxygen needed for optimal chemotherapy or radiation therapy and also lowering the systemic levels of tyrosine, thereby delaying the growth of the melanoma.

We used glutaraldehyde to crosslink hemoglobin and tyrosinase and to optimize the preparation to provide, typically, a hemoglobin : tyrosinase molar ratio of 100 : 2 (Yu and Chang, 2004a). Characterization includes the enzyme kinetics (K_m, V_{max}, pH optimal and stability, temperature stability at 0°, 20° and 37°C), and molecular weight distribution. The optimized preparation retains 95–99 per cent of the original tyrosinase activity. It also retains the oxygen-carrying ability of PolyHb (Figure 38.8). Animal studies have shown that the novel polyhemoglobin–tyrosinase preparation can rapidly lower the body tyrosine level after a single intravenous injection to 5 per cent of control level in 1 hour (e.g. 1.598 ± 0.320 mg/dl decreased to 0.077 ± 0.022 mg/dl). However, the lowered levels are only maintained for up to 1 day.

B16-F10 murine melanoma cells (American Type Tissue Collection, Manassas, VA, USA) were cultured in complete DMEM until they became 30–40 per cent confluent. Then, one of the following four solutions was added to the culture medium: (1) saline solution (0.9 g/dl NaCl); (2) free tyrosinase solution; (3) PolyHb solution and (4) PolyHb–tyrosinase solution. Table 38.1 shows that PolyHb by itself did not have any effects on the growth of the B16F10 cells when compared to saline. Tyrosinase in the complex had similar inhibition effects to the free tyrosinase on the growth of the melanoma cell culture (Yu and Chang, 2004b; Table 38.1). Tyrosinase in the free form was removed rapidly from the circulation whereas tyrosinase in PolyHb–tyrosinase retained the same circulation time as polyhemoglobin. Furthermore, tyrosinase in PolyHb– tyrosinase is covered and protected from being exposed to the body by PolyHb with a hemoglobin : tyrosinase molar ratio of 100 : 2.

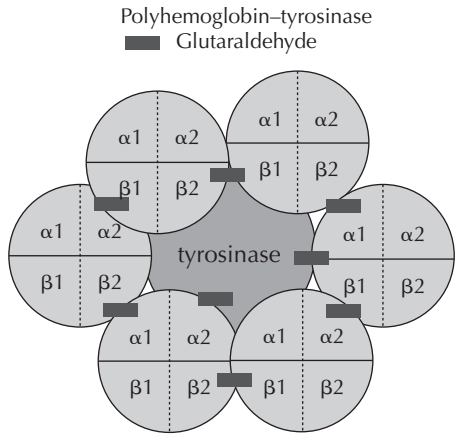

Figure 38.7 Polyhemoglobin-tyrosinase complex formed by glutaraldehyde crosslinking (from Yu and Chang, 2004a).

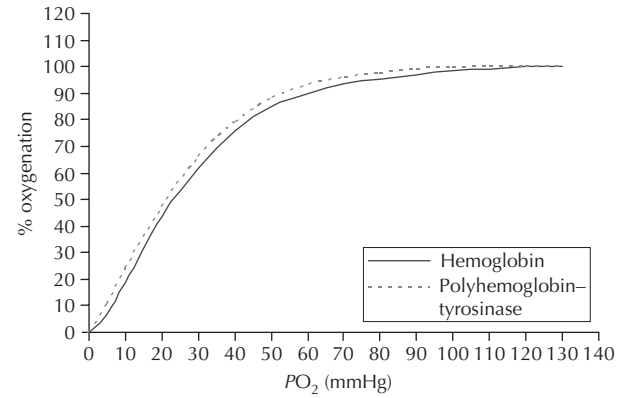

Figure 38.8 Oxygen dissociation curve of polyhemoglobin and polyhemoglobin-tyrosinase (modified from Yu and Chang, 2004b).

Table 38.1 B16F10 melanoma cell numbers (1×10^5) in cell culture after the addition of saline, PolyHb, free tyrosinase or PolyHb–tyrosinase to the medium (Yu and Chang, 2004b)

Day	Saline	PolyHb	Free tyrosinase	PolyHb–tyrosinase
0	0.7 ± 0.1	0.7 ± 0.04	0.8 ± 0.05	0.8 ± 0.1
4	29.5 ± 1.7	28.6 ± 3.9	0.4 ± 0.06	0.3 ± 0.03

BD2F1 female mice (C57BL/6 × DBA/2F1) at age 57–63 days were used. Two groups of mice were studied. The control group received daily 0.1 ml intravenous injections of saline and the test group received daily intravenous injections of 0.1 ml of PolyHb–tyrosinase solution. PolyHb–tyrosinase reduced plasma tyrosine levels rapidly to 0.19 ± 0.09 mg/dl on day 2, compared to 1.44 ± 0.16 mg/dl in the control saline group (Yu and Chang, 2004b). The systemic tyrosine concentration was kept at a low level by continuing with this daily injection of PolyHb–tyrosinase. Daily measurements of body weight showed no difference between the control group and the test group. Furthermore, we note no loss of appetite or vomiting in these mice.

B16F10 melanoma cells prepared at 1×10^6 in 0.1 ml of HBSS were injected subcutaneously into a shaved lateral flank of the mice. The sizes of primary tumors were measured every 2 days using calipers. Tumor volume was calculated using the formula $V = (A \times B^2)/2$, where V was the volume (mm^3), A the length (mm) and B the width (mm) of the tumor (Barthelmes *et al.*, 2001). When the tumor volume reached an average of 125 mm^3 on day 9, we started one of the following regimes: (1) the sham control group received no intravenous injections; (2) the saline group received daily intravenous injections of 0.1 ml saline; (3) the PolyHb–tyrosinase group received daily intravenous injections of 0.1 ml of PolyHb–tyrosinase solution. The endpoint of this study was based on the Faculty of Medicine Animal Care Committee's regulation that tumor burden should not exceed 10 per cent of the animal's normal body weight. Table 28.2 and Figure 38.9 show that there was no significant difference in tumor size between the sham control group and the saline group. On the other hand, on day 4 of the daily intravenous injections of PolyHb–tyrosinase, the tumor volume was significantly lower than in the saline group. On day 6 of the daily intravenous injections, the tumor volume in the PolyHb–tyrosinase group was only 53 ± 14 per cent of that in the saline group. Nineteen days after the inoculation of the B16F10 melanoma cells, the tumor volume of the control had reached the maximal of 10 per cent of body weight allowed by the Animal Care Committee and we had to terminate the study. At this time, the tumor size in the PolyHb–tyrosinase group was only 45 ± 10 per cent of that of the saline group (Yu and Chang 2004b). Therefore, our results suggest that PolyHb–tyrosinase retards the growth of B16F10 melanoma in mice. We also followed the body weight of these three groups of mice, and there was no significant difference in weight gain (Yu and Chang, 2004b).

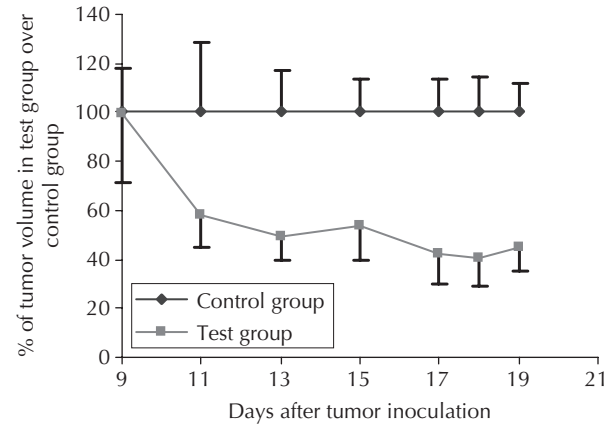

Figure 38.9 Tumor growth of B16F10 melanoma in mice shown as percentage of tumor volume in test group over control group. (1) Saline control, 0.1 ml intravenous saline daily; (2) test group, 0.1ml intravenous PolyHb–tyrosinase daily. Endpoint of study was when tumor in the control group reaches 10 per cent of normal body weight on day 19 (Faculty of Medicine Animal Care Committee regulations). All values are represented as mean ± SEM (modified from Yu and Chang, 2004b).

Table 38.2 Tumor growth (mm^3) of B16F10 melanoma in mice. Nine days after implantation of B16F10 melanoma, one of the following three regimens was followed: (1) sham control, no intravenous injection; (2) saline, 0.1 ml intravenous saline daily; (3) PolyHb–tyrosinase, 0.1 ml intravenous PolyHb–tyrosinase daily. All values are mean ± SEM. (Yu and Chang, 2004b)

Day	Sham control (mm^3)	Saline (mm^3)	PolyHb–tyrosinase (mm^3)
9	123 ± 33	125 ± 24	124 ± 35
19	3263 ± 259	3190 ± 367	1444 ± 322

SUMMARY

There are many conditions that need oxygen carriers. Thus polyhemoglobin, conjugated hemoglobin and other oxygen carriers are useful in conditions that are not predisposed to ischemia-reperfusion injuries. Even in those conditions with potential for ischemia-reperfusion injuries, it is important to know the duration and severity of ischemia. In this regard, much research will be needed to analyze the criteria for using polyhemoglobin–SOD–catalase. In the case of polyhemoglobin–tyrosinase, our results encourage further studies to optimize this further and to investigate the combined use of polyhemoglobin–tyrosinase with radiation therapy or chemotherapy for treating malignant melanoma.

EDITOR'S SUMMARY

One theory to explain the toxicity observed in clinical and animal trials with cell-free hemoglobin is its activity as an oxidant, which can lead to the production of oxygen (and other) free radicals. Inside the red blood cell, any such toxic molecules are reduced by enzyme systems, including superoxide dismutase (SOD) and catalase. This chapter explores a number of interesting experiments using hemoglobin first polymerized with glutaraldehyde, then crosslinked to SOD. The product transports oxygen, but also provides the reducing power of SOD. This product has not yet reached clinical trials, but could be very promising in some clinical indications.

A completely separate indication is addressed by glutaraldehyde-polymerized hemoglobin crosslinked to tyrosinase. This product is not primarily an oxygen therapeutic or blood substitute, but rather uses hemoglobin as a carrier for tyrosinase, which has demonstrated activity against melanoma cells. Experiments in cell culture and animal studies suggest that this approach might have important clinical utility, but it has not been developed as yet.

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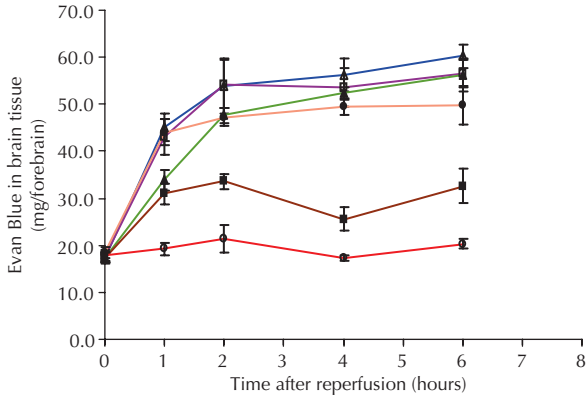

Plate 16 Effects of PolyHb–SOD–CAT on blood–brain barrier. PolyHb–SOD–CAT significantly attenuated the severity of blood–brain barrier disruption as compared to reperfusion with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb. $P < 0.01$. (From Powanda and Chang, 2002, with permission.) See Fig. 38.5.

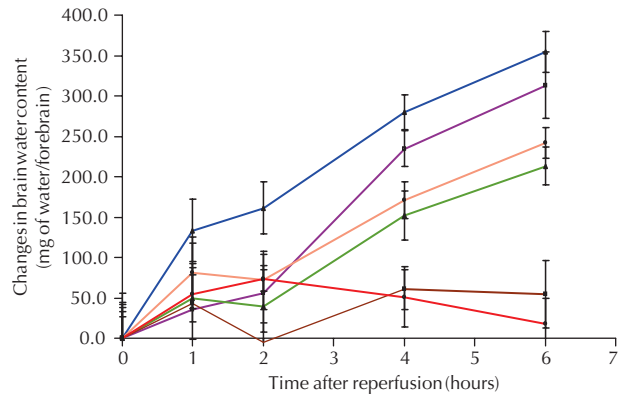

Plate 17 Brain edema: changes in brain water content. The changes in brain water content of PolyHb–SOD–CAT treated animals were not significantly different from that of the sham control. The increase in water content when reperfused with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb was significantly different from that of the sham control and PolyHb–SOD–CAT group by the fourth hour and increased there on with time. $P < 0.01$. (From Powanda and Chang, 2002, with permission. See Fig. 38.6.

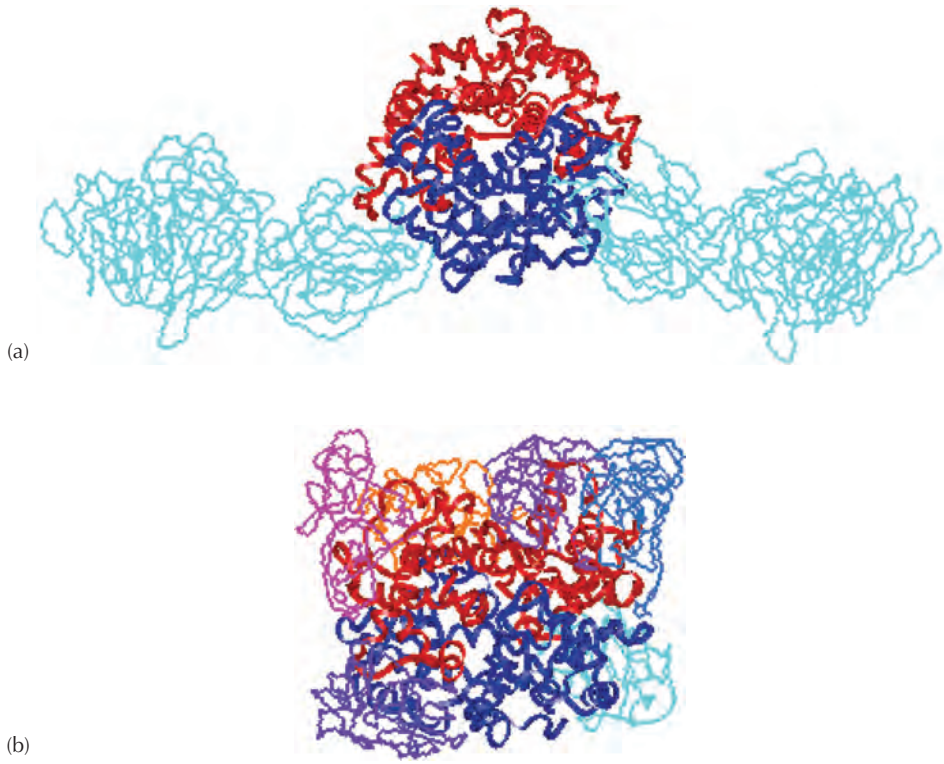

Plate 18 Molecular models of hemoglobin with two PEG 20 000 chains and six PEG 5000 chains. See Fig. 39.4.

Chapter 39

Surface Decoration of Hemoglobin with Polyethylene Glycol

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PROTEIN MODIFICATION WITH PEG

Conjugation of polymers to peptide and protein therapeutics to generate hybrid molecules with unique and distinct molecular properties has become a popular approach to alter and/or control their stability, biodistribution, pharmacokinetics and toxicology. Since the pioneering work of Abuchowski *et al*. (1977a), grafting of polyethylene glycol (PEG) chains to proteins (PEGylation) is one of the most widely used conjugation approaches. PEG reagents of various sizes have been used for PEGylation. Although linear PEG reagents of various molecular sizes have been the choice molecules in the earlier studies, branched PEGs and very large linear PEG chains are now available, especially for site-specific PEGylation (Bailon *et al.*, 2001; Roberts *et al.*, 2002). PEGylation using these new large and/or branched PEGs has been referred to as advanced PEGylation. Polyethylene glycols are inert and nontoxic polymers. Accordingly, there has been significant interest in developing PEGylated protein therapeutics for clinical applications.

Conjugation of the PEG chains to bovine serum albumin using 2,4,6-trichloro triazine (cyanuric chloride) activated PEG was the first study of modifying the protein with PEG, and multiple copies of PEG chains were attached to the protein. PEGylated bovine serum albumin (Abuchowski *et al.*, 1977a) is incapable of eliciting antibody to itself or the unmodified albumin, i.e., PEGylation of the protein has enabled it to camouflage itself from the immune system. Furthermore, the PEGylated albumin showed extended circulating life in the blood. The same approach was used to conjugate PEG chains to enzymes, catalase being one of the first enzymes to be PEGylated (Abuchowski *et al.*, 1977b). Several other proteins were subsequently modified with PEG. Most of the PEGylated protein therapeutics carry a single copy of the PEG chain of desired molecular size. PEGylation of protein therapeutics improves their clinical properties, increases their absorption, and reduces immunogenicity and proteolysis, and all these beneficial effects of PEGylation contribute to the increase in the efficacy of the therapeutic protein as compared to the parent protein (Nucci *et al.*, 1991; Bailon and Berthold, 1998; Pasut *et al.*, 2004). Some of the therapeutic proteins the PEGylated versions of which are either in clinical trial or in clinical use are asparaginase, interferon, interleukin, tumor necrosis factor and granulocyte macrophage colony stimulating factor.

HEMOGLOBIN MODIFICATION WITH PEG

The development of a safe and effective hemoglobin-based oxygen carrier has faced

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many challenges over the years (Winslow, 1999; Reiss, 2001). The early design strategies for the preparation of HBOCs focused on eliminating the nephrotoxicity of acellular Hb. These strategies can be broadly classified into three groups: (1) stabilization of the tetrameric structure of Hb by intramolecular crosslinking; (2) increasing the molecular size of Hb by inter-tetrameric crosslinking (oligomerization/polymerization); and (3) increasing the molecular size of Hb by conjugation with inert polymers such as dextran, polyvinyl sulfone and PEG. Although several of the approaches have not been fully successful, they have contributed profoundly to our understanding of the characteristics desired in such a product. This chapter reviews:

- the development of the PEGylation of Hb as a strategy to overcome the nephrotoxicity of acellular Hb
- the recognition that PEGylated Hb (with multiple copies of PEG chains) possibly functions as an oxygen-carrying plasma expander
- the development of a new thiolation-mediated maleimide chemistry-based PEGylation protocol for the surface decoration of Hb
- the demonstration that surface decoration of Hb with multiple copies of small PEG chains is a preferred approach over the conjugation of a small number of large PEG chains for the neutralization of the vasoactivity of Hb.

Conjugation of hemoglobin with PEG chains

The observation that the conjugation of PEG to proteins increases their circulatory half-life and reduces kidney filtration, antigenicity, and immunogenicity led to the application of this process for the PEGylation of Hb. PEG contains two terminal hydroxyl groups that can be chemically activated. Typically, PEGylation of proteins involves activating PEG with a functional group which will react with the side chain functions of the protein. In general, in the preparation of mono-functional PEG reagents, one of the two hydroxyl groups of the PEG is converted to a methoxy group and the other one is chemically activated with the desired reactive moiety. By an appropriate choice of the reactive moiety of the PEG reagent, it is possible to conjugate the PEG at specific sites of the protein (for example, at sulfhydryl, amino or carboxyl) under mild, physiological reaction conditions, and without the use of toxic chemicals (Roberts *et al*., 2002; Caliceti and Veronese, 2003).

While PEG reagents having one reactive moiety would generate surface-decorated protein, the PEG reagents with reactive moieties at both the terminal hydroxyls (bi-functional PEG) would provide a means for generating dimeric and multimeric proteins by crosslinking two side-chain functions of the protein. Thus, the same reaction chemistry could be used to generate both surface-decorated Hb and multimeric Hbs.

Although there are several examples of PEGylation of Hb (Nho *et al*., 1992), the most well studied are the PEGylated bovine Hb of Enzon (Zalipsky *et* al., 1991; Nho *et al*., 1994; Shorr *et al*., 1995) and the PEGylated pyridoxalated Hb (PHP) of Ajinomoto (Iwashita *et al*., 1988), now licensed to Apex Biosciences (Talarico *et al*., 1998, 2000). In both cases, the PEGylation reaction is targeted to the surface amino groups of Hb and the acylation of the amino groups is accomplished using succinimidyl esters of PEG. In the process developed by Enzon, a PEG-succinimidyl carbonate (SC-PEG) is reacted with the lysine amino groups of bovine Hb resulting in the conjugation of PEG to the protein through a urethane linkage (Figure 39.1). Reaction of bovine Hb with SC-PEG of molecular weight ranging from 2000 to 5000 has been reported (Zalipsky *et al*., 1991), the most studied preparation being the one generated using SC-PEG 5000 (Shorr *et al*., 1995).

In the protocol developed by Ajinomoto (Iwashita *et al*., 1988), the PEGylation of the surface amino groups of pyridoxalated human Hb is accomplished using a bi-functional PEG succinimidyl active ester, bSOA-PEG3600, in the

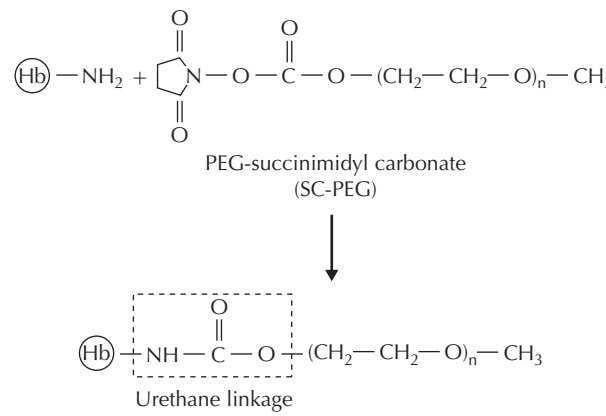
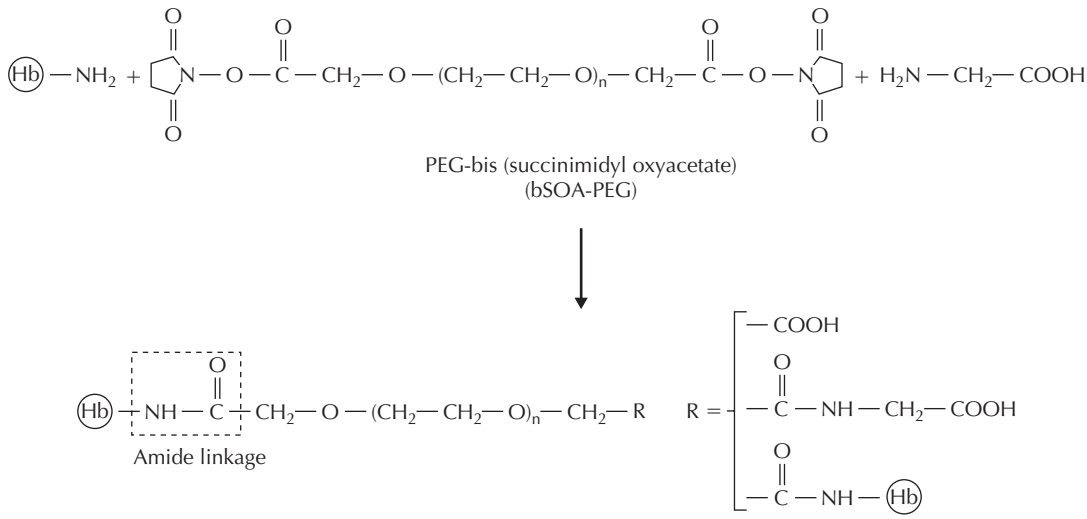


Figure 39.1 Schematic representation of the chemistry of conjugation of SC-PEG to hemoglobin.



presence of glycine (Figure 39.2). In this example, the PEG chains are bound to the hemoglobin molecule through an amide linkage. The PEG-Hb thus produced consisted of 90 per cent unpolymerized and 10 per cent polymerized protein. Furthermore, as a consequence of the use of a bifunctional reagent in the presence of a low molecular weight amino acid component, besides generating intra- and intermolecular crosslinks, this process also results in the surface decoration of the intra- or intermolecularly crosslinked Hbs in two modes: (1) the succinimidyl ester at the distal end (i.e., the functionalized end that has not reacted with Hb) is hydrolyzed to a free acid or (2) it reacts with glycine. Thus this product is expected to be very heterogeneous.

VASOACTIVITY OF PEG-MODIFIED HEMOGLOBIN

Preclinical data using a number of potential Hb-based oxygen carriers have demonstrated hypertension as the most common and serious toxic effect of these products (Hess *et al.*, 1993; Thompson *et al.*, 1994). Overcoming the vasoactivity of acellular Hb has been the major obstacle in realizing the potential therapeutic application of the Hb-based oxygen carriers. The vasoactivity of Hb has, at least in part, been attributed to its nitric oxide scavenging activity. Two main approaches advanced to overcome the vasoactivity of Hb based oxygen carrier are (1) generation of mutant Hbs with low NO binding activity (Doherty *et al.*, 1998), and (2) increasing the size of Hb by oligomerization using crosslinking reagents, which will reduce the extravasation of Hb from the vascular space where it can scavenge NO. A recent study by Rohlfs *et al.* (1998) demonstrated that, although intramolecularly crosslinked, oligomerized and PEG-modified bovine Hb exhibited nearly identical NO binding activity, the PEG-modified bovine Hb was non-hypertensive. One or more structural and or functional consequences of PEG modification appear to overcome the vasoconstrictive activity of bovine Hb. The PEG-modified hemoglobin used in the Rohlfs study was produced by Enzon. Since it consists of bovine hemoglobin conjugated with 10 strands of PEG 5000, according to the nomenclature used in this chapter, it will be denoted Hbv-PEG5K10.

NEW PARADIGMS FOR THE DESIGN OF HEMOGLOBIN-BASED BLOOD SUBSTITUTES

Critical properties revisited

Hemodilution with conventional colloids does not induce vasoconstriction. On the other hand, hemodilution with most of the Hb-based oxygen carriers, except Hbv-PEG5K10, causes varying degrees of hypertension. The colloidal osmotic pressure and viscosity of Hbv-PEG5K10 are higher than those of other Hb-based oxygen carriers but comparable to those of many plasma

expanders in clinical use, such as the starches. This comparison suggested that the paradigm for the design of Hb-based oxygen carriers is to endow acellular Hb higher viscosity and colloidal osmotic pressure.

The above studies led to a re-evaluation of the design strategies for the development of safe and effective HBOCs (Tsai and Intaglietta, 2002; Winslow, 2004). Based on the results of the microcirculation studies of Hb solutions and many plasma expanders, and the recognition of the role of autoregulatory mechanisms in maintaining the vascular tone *in vivo*, the observations with the BvHb-PEG5K10 led to the recognition of new paradigms for the design of blood substitutes:

1. Increase the oxygen affinity of Hb to limit the oxygen off-loading by Hb and attenuate the oxygen tension mediated vasoconstriction in highly vasoactive arterioles.
2. Increase the viscosity of the hemoglobin solution to reduce the diffusion constants for oxyhemoglobin and to maintain or slightly increase the shear stress on the vasculature after Hb-based oxygen carrier is infused into the circulatory system.
3. Increase the colloidal osmotic pressure of the potential blood substitutes comparable or superior to blood so that the functional capillary density is maintained at a high level after infusion of the test material. An increase in the colloidal osmotic pressure shifts the interstitial fluid balance between the extravascular and vascular spaces. The combination of increased viscosity and higher blood flow rate amplifies the shear stress on the endothelial wall, and this in turn can promote the release of vasodilatory factors.
4. Increase the molecular volume of Hb to decrease the extravasation of the Hb-based oxygen carrier from the vascular space to extravascular space.

Validation of the new strategies

The lack of hypertension exhibited by infusion of BvHb-PEG5K10 was attributed to PEGylation, which endowed the Hb molecule with high viscosity, high oncotic pressure, and high molecular volume (Vandegriff *et al.*, 1997a), i.e., properties of colloidal plasma expanders. Given the present knowledge of the autoregulation of blood pressure through the oxygen tension in the arteries, a higher oxygen affinity of the blood substitute appears to be the preferred property to generate a non-hypertensive Hb. PEGylation of Hb that generates a high oxygen affinity and high molecular volume with the desired viscosity and colloidal osmotic pressure appears to be the strategy for the design of non-hypertensive Hb. It is also conceivable that the generation of non-hypertensive bovine Hb on PEGylation could be unique either to bovine Hb or to the chemistry of conjugation.

NEW PEG CHEMISTRY STRATEGIES

With a view to validating the beneficial effects of surface decoration of Hb with PEG, we have designed a new PEGylation protocol that uses monofunctional maleimide PEG 5000 as the PEGylating reagent to target the PEGylation to the surface of the amino groups of Hb.

Thiolation-mediated maleimide chemistry

Of the many group-specific chemical modification reactions of proteins, the reaction of maleimides with the thiol groups of Cysteine residues of proteins is a very simple and highly specific reaction; and generally the modification of protein can be carried out at neutral pH in the cold with minimal or no side reactions. Hence, this chemistry has the potential to yield products with greater homogeneity. Although Hb has six Cysteine residues per tetramer (three per $\alpha\beta$-dimer), none are reactive in the deoxy conformation. However two, namely Cysteine 93(β), are reactive in the oxy conformation. Thus, the reaction of oxyHb with the PEG maleimide results in the conjugation of only two copies of PEG 5000 chains per Hb.

Accordingly, in order to generate a PEGylated Hb with multiple copies of PEG 5000 chains using the maleimide PEG 5000, new thiol groups need to be engineered into Hb. This can be achieved either through site-directed mutagenesis by introducing Cysteine residues at preselected sites on the molecular surface of Hb, or through chemical modification of Hb that introduces thiol groups on the exposed amino groups of Hb. Our initial choice has been the chemical modification approach targeting the thiolation to the ε-amino groups of the surface Lysine residues of Hb, and in a conservative mode, i.e. without altering the positive charge of the amino groups. This can be achieved by reaction of the protein with 2-iminothiolane (2-IT) (Acharya *et al.*, 1996; see Figure 39.3). Iminothiolane is a

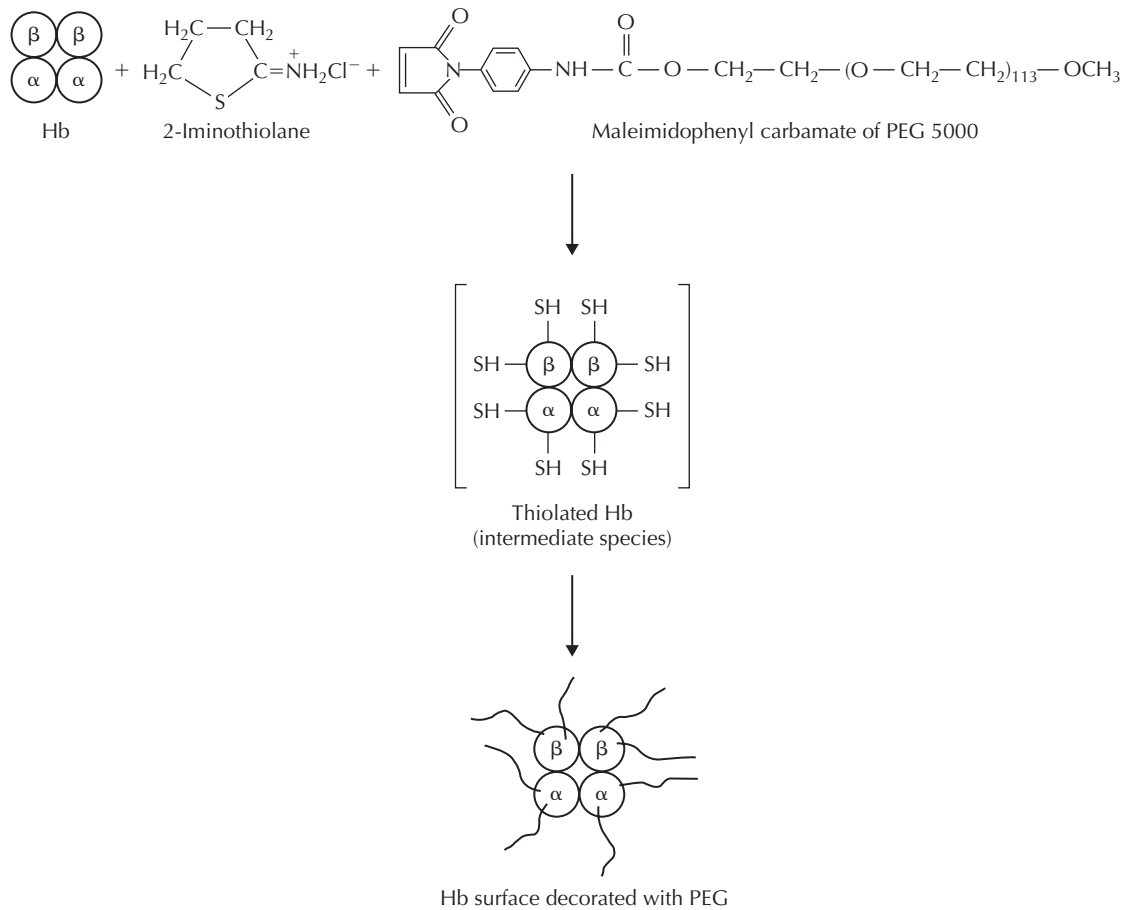


Figure 39.3 Schematic representation of the thiolation-mediated maleimide chemistry based PEGylation of Hb.

small molecular weight reagent, a cyclized product of δ-mercapto butyrimidate. Accordingly, 2-IT by itself does not carry a free thiol group, and generates a thiol group *in situ* only after reaction with the amino group. On reaction with 2-IT the ε-amino group of the protein is converted to a substituted amidine, resulting in the introduction of δ-mercapto butyrimidyl chain on the reactive surface amino groups. In this conversion, the positive charge of the ε-amino groups of Hb is conserved. In the PEGylated Hb, the δ-mercapto butyrimidyl moiety serves as an extension arm between Hb and the PEG. Thus, the conjugating linkage between the PEG and Hb in the product generated with this protocol is very distinct from the urethane linkage present between the PEG and bovine Hb in BvHb-PEG5K10. Furthermore, the formation of the urethane linkage neutralizes the positive charge at the site of conjugation. This difference in the chemistry of conjugation could influence the structural, functional and vasoconstrictive properties of Hb.

Since 2-IT by itself does not contain a free sulfhydryl group, and is generated *in situ* on reaction of 2-IT with Hb, the PEGylation could be carried out by incubating Hb simultaneously with 2-IT and the desired maleimide PEG (Figure 39.3). The extent of thiolation of Hb could be controlled by the molar excess of 2-IT over Hb in the reaction mixture. Thus, the extent of thiolation determines the extent of PEGylation. This new PEGylation protocol has been optimized to produce a PEGylated Hb that carries an average of six copies of PEG 5000 chains per molecule (Hb-PEG5K6). Of the six PEG chains, two are on Cysteine 93(β) and the remaining four are distributed on a limited number of the surface ε-amino groups of the protein.

Structural and functional properties of Hb-PEG5K6

Although the calculated molecular mass of Hb-PEG5K6 is only around 94 kDa, its hydrodynamic

volume is comparable to a globular protein of molecular size of 260–300 kDa. Thus, the surface decoration of HbA with PEG 5000 increases the apparent molecular volume of the protein considerably more than the actual mass of the PEG covalently linked to the protein. The increase in the hydrodynamic volume of Hb as a result of PEGylation is nearly six to eight times higher than that expected from the conjugation of a globular protein of comparable mass. Thus, the Hb-PEG5K6 is a size-enhanced molecule, comparable to that of oligomerized Hb consisting of four to five Hb tetramers (Acharya *et al*., 2003).

Although Hb-PEG5K6 elutes in size exclusion chromatography as a symmetrical peak suggesting molecular size (hydrodynamic volume) homogeneity, globin chain analysis and tryptic peptide mapping of Hb-PEG5K6 has established that it is composed of a mixture of positional isomers in terms of the sites of PEGylation (Manjula *et al*., unpublished results).

Compared to native Hb, Hb-PEG5K6 has increased oxygen affinity and reduced sensitivity to the allosteric effectors 2,3-DPG, H^+ and chloride. The degree of increase in the oxygen affinity of Hb-PEG5K6 appears to be a function of the ionic strength of the buffer used for its measurement; the oxygen affinity of Hb varies with ionic strength, but this is not the case with Hb-PEG5K6 (Manjula *et al*., unpublished results). This suggests that the structure of the 'PEG shell' around the Hb molecule insulates it from changes in the macroenvironment.

The subunit surface conformation of Hb is not significantly altered in Hb-PEG5K6. The hydrogen bonding pattern at the subunit interfaces at the $\alpha_1\beta_1$ and the $\alpha_1\beta_2$ interfaces is comparable to that of unmodified Hb in both the oxy and the deoxy conformations. The geminate recombination studies of Hb modified at Cysteine 93(β) with N-ethyl maleimide (NEM) and Hb-PEG5K6 have demonstrated that the maleimide-based modification induces the maximum influence on the *R*-state conformation of Hb. The altered *R*-state conformation is reflected in reduced geminate yield and loss of the heme–proximal histidine interaction normally seen for the liganded *R*-state HbA. The results can be explained on the basis of the displacement of the side chain of Tyrosine 145(β) as well as other situations where there is loss of quaternary enhancement (Khan *et al*., 2001). The resonance Raman spectroscopic investigations of Hb-PEG5K6 suggest a perturbation of the deoxy state by 'loosening' of the contacts associated with the switch region of the *T*-state $\alpha_1\beta_2$-interface, but does not modify the hinge region of this interface. The surface-decorated Hbs exhibit general intensity enhancements of the tyrosine and tryptophan bands in the UV resonance Raman spectrum (Juszczak *et al*., 2002). It is suggested that this effect arises from the osmotic impact of the large PEG molecules enveloping the surface of Hb.

The molecular radius of Hb-PEG5K6 is around 6.5 nm as compared to the value of 3.0 nm for unmodified Hb. Thus the molecular volume of Hb-PEG5K6 is increased by nearly eight to nine times that of Hb. Based on colloidal osmotic pressure measurements, Vandegriff *et al*. (1997a, 1997b) calculated that the molecular radius of the Enzon PEG-modified bovine hemoglobin is $\sim$15 nm, and that it carries $\sim$ 10 copies of PEG 5000 per Hb (thus BvHb-PEG5K10). The size enhancement achieved with Hb-PEG5K6 appears to be less than that of the BvHb-PEG5K10. Both the viscosity and the colloidal osmotic pressure increase exponentially with protein concentration in Hb-PEG5K6.

Vasoactivity of Hb-PEG5K6

The vasoactivity of Hb-PEG5K6 has been investigated in a hamster window chamber model using a 10 per cent top-load infusion, and also in 50 per cent exchange transfusion experiments in rats (Acharya *et al*., 2005). In the hamster system, the functional capillary density remained comparable to that of the untreated controls and arteriolar diameter remained essentially unaffected, whereas the animals infused with Hb exhibited a significant decrease in the functional capillary density and the arteriolar diameter. Thus PEGylation of Hb neutralizes the vasoconstrictive activity of acellular Hb. Similarly, the rats exchange-transfused with Hb-PEG5K6 did not show any significant increase in the mean arterial pressure, demonstrating the neutralization of the hypertensive activity as well. These results independently established that surface decoration of Hb with PEG increases its hydrodynamic volume (apparent molecular size), viscosity and colloidal osmotic pressure, and makes the Hb non-hypertensive. Thus it appears that a unique combination of one or more of the properties of PEGylated Hb, endowed to the protein on PEGylation, neutralizes the propensity of acellular Hb to invoke vasoactivity.

Flexibility of the thiolation-mediated maleimide chemistry-based PEGylation protocol

One of the unique features of the new PEGylation protocol developed is the high flexibility. The conservative PEGylation protocol as discussed above is carried out as a one-step procedure, wherein Hb is incubated simultaneously with both 2-IT and Mal-PEG. In this protocol, the thiol groups generated *in situ* are immediately trapped by the Mal-PEG. Thus, the likelihood of the nascent thiol groups forming disulfide bonds to generate inter-tetrameric crosslinks is negligible, even though the reaction is carried out under oxy conditions. In our hands, when the two reactions are carried out sequentially, some inter-tetrameric crosslinks are generated, increasing the chemical heterogeneity of the product.

The other flexibility of the protocol is that the number of the PEG chains introduced onto the Hb molecule can be readily controlled by changing the concentration of 2-IT in the reaction mixture (i.e., number of the thiol groups incorporated into Hb). The size enhancement of the Hb molecule could be controlled by this approach (i.e., by controlling the number of PEG chains that can be conjugated), or by changing the molecular size of the Mal-PEG used (3000, 5000, 10 000 or 20 000 Da PEG), or by changing both the concentration of 2-IT and the molecular size of the Mal-PEG.

A variety of Mal-PEGs can be used for the reaction, since the specificity of the PEGylation is dictated by the maleimide chemistry. We routinely use Mal-phenyl PEG, since the functionalizing strategy used in synthesizing this type of Mal-PEG is a one-step reaction and hence a reagent of higher purity is obtained (one of the primary impurities in the PEG reagents appears to be the peroxy ethers). A multi-step activation enhances the likelihood of the generation of peroxy ether in the PEG reagent, which leads to the production of met Hb in the PEGylated Hb.

We have used 2-IT mediated thiolation with ethyl Mal-PEG as well to produce the Hb-PEG5K6 and have investigated the influence of the linker arm on the biophysical properties of the product (Khan *et al*., 2001; Juszczak *et al*., 2002). Vandegriff *et al*. (2003) have used the 2-IT mediated thiolation reaction with propyl Mal-PEG 5000 to generate a Hb-PEG5K6 (MP4, see Chapter 40) which is also non-hypertensive.

MODIFICATION NEEDED TO NEUTRALIZE VASOACTIVITY

The reaction of oxyHb with phenyl Mal-PEG 5000 in the absence of 2-IT results in Hb-PEG5K2, a molecule with two copies of PEG-5000, one on each of the two Cysteine 93(β) residues (Manjula *et al*., 2000, 2003). The hydrodynamic volume of this product is comparable to that of a globular protein of molecular size 125 000 Da, even though its actual molecular mass is only around 75 000 Da. Its oxygen affinity is comparable to that of the Hb-PEG5K6.

Hb-PEG5K2 produces vasoconstriction after a 10 per cent top-load in the hamster, whereas Hb-PEG5K6 does not. The oxygen affinities of the two molecules are comparable, but Hb-PEG5K2 has lower molecular volume, lower viscosity and lower colloidal osmotic pressure. The Enzon BvHb-PEG5K10 is also non-hypertensive, but the PEG-5000 chains are covalently linked to surface amino groups with urethane linkages (Vandegriff *et al*., 1997b), resulting in the loss of the positive charge of the derivatized amino group. Whether or not the conservation of the positive charge contributes to physiologic properties is not apparent at present.

The hydrodynamic volume, viscosity and colloidal osmotic pressure could also be increased to a level comparable to that of the Hb-PEG5K6 by attaching fewer longer-chain PEGs at Cysteine 93(β). Indeed, Hb-PEG20K2 exhibits molecular radius, hydrodynamic volume, viscosity and colloidal osmotic pressure close to that of Hb-PEG5K6; however, it is more vasoactive than Hb-PEG5K6, suggesting that the pattern of conjugation (the number and size of the PEG chains) of the PEGylated Hb influences the neutralization of vasoactivity.

MOLECULAR SHIELDING BY PEG MODIFICATION

Both Hb-PEG5K6 and Hb-PEG20K2 satisfy the new paradigm of the design of blood substitutes (high oxygen affinity, high oncotic pressure, high viscosity and high hydrodynamic volume), yet they have different vasoactivities. It is possible that the propensity of PEG chains to generate a PEG 'shell' around Hb that can insulate the molecule from the bulk water is distinct between the two PEGylated Hbs. To gain insight into this

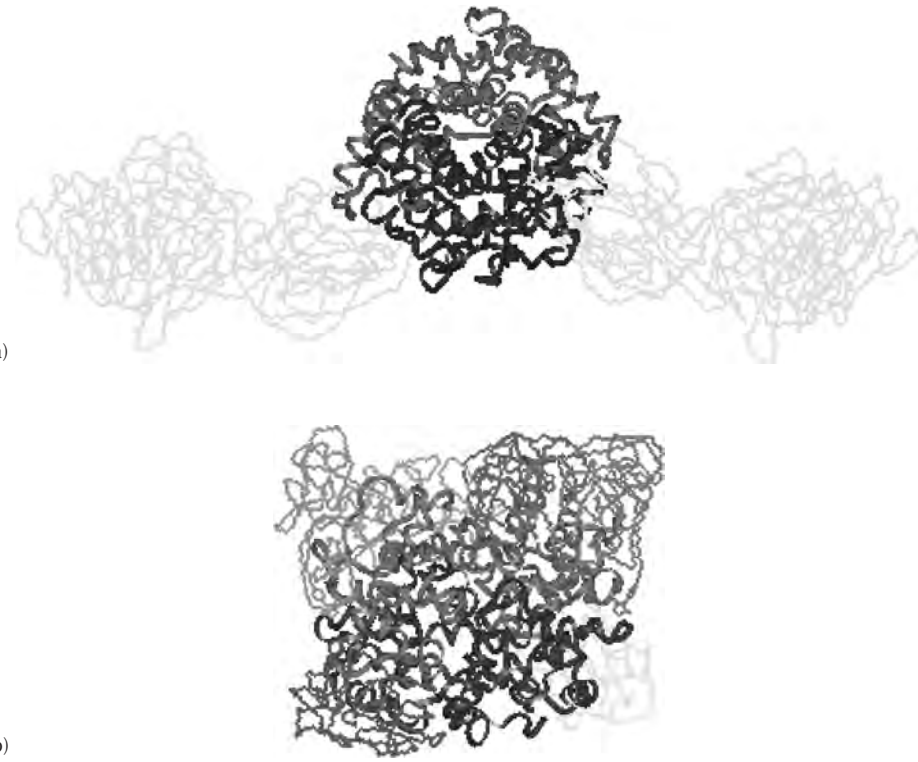


Figure 39.4 Molecular models of hemoglobin with two PEG 20 000 chains and six PEG 5000 chains. See color plate 18.

concept, we have generated molecular models of these PEG-Hbs (Figure 39.4). This analysis suggests that six copies of PEG-5000 afford a better surface coverage than two copies of PEG-20 000, possibly because of greater uniformity of the shell in the case of the shorter PEG chains. The biophysical significance of a PEG shell and its relation to vasoactivity of cell-free Hb is not clear at the moment, but it is expected to be a subject of future investigations.

From this discussion, it would appear that 10 copies of PEG 5000 per Hb tetramer, as in the case of Enzon's BvHb-PEG5K10, are not needed to overcome vasoactivity. It could be speculated that in increasing the size of the PEG shell, a 'saturation point' is reached when a minimal number of PEG chains are covalently attached to the protein. Our data suggest that the saturation point for the radius of the PEGylated Hb is around 6–7 nm when PEG 5000 is used as the decorating PEG. Once this size is reached, conjugation of additional PEG strands on the PEGylated protein becomes rather inefficient, even though the protein could be modified further (Acharya, unpublished results).

SUMMARY

PEG modification of hemoglobin is a versatile process that lends itself to a variety of manipulations. The ability to choose the target site on the protein by the appropriate choice of functionalized PEG (the chemistry of conjugation) and the ability to choose the size of the PEG in a given functionalized PEG allows for a variation of the site of conjugation, and the size and number of PEG molecules conjugated. We have generated Hb-PEG5K6 using other PEGylation chemistries, thiocarbamoylation, acylation and reductive alkylation using the respective functionalized PEG 5000 chains for these chemistries (Acharya *et al.*, unpublished results). A systematic comparative evaluation of the colligative properties, hydrodynamic volume, viscosity and molecular radius of the PEGylated Hb with different conjugation chemistries is essential to dissect out the correlation of the PEGylation-induced properties of surface-decorated Hb with the neutralization of the hypertensive/vasoconstrictive activity of acellular Hb.

The results of preclinical and clinical studies carried out with another version of the

hexaPEGylated Hb produced based on the thiolation-mediated maleimide chemistry-based PEGylation protocol described in the present study are very encouraging for its potential as a successful HBOC candidate (Winslow, 2004). In light of the feasibility of modulating the molecular dimensions and the solution properties of the PEGylated Hb by manipulation of the chemistry of PEG conjugation (site and chemistry of conjugation and the size and number of PEG chains), PEGylation of hemoglobin appears to hold promise for the development of a safe and effective oxygen-carrying plasma expander.

EDITOR'S SUMMARY

Increasing the size of the hemoglobin molecule appears to lead to a reduction in one of its key sources of toxicity: vasoconstriction. Several different approaches support this claim, including polymerizing recombinant hemoglobin (Baxter), polymerizing hemoglobin itself (Bucci), conjugation to dextran (Wong) or surface modification with polyethylene glycol (PEG).

Newer and novel chemistry, using attachment of maleimide-activated PEG to surface thiol groups on hemoglobin, allows exploration of biophysical properties such as surface charge, hydrodynamic volume, oncotic pressure, oxygen affinity and viscosity in systematic ways. Properties of hemoglobin with two chains of 20-kDa PEG compared with six chains of 5-kDa PEG show a difference in biological activity (i.e., vasoconstriction), further suggesting that the uniformity of the PEG 'shield' covering the hemoglobin molecule may be an additional important property.

Using this systematic approach, it should be possible to optimize the surface modification of hemoglobin to reduce or eliminate its vasoactivity while optimizing the reaction conditions and minimizing the amount of PEG (and therefore cost) of production.

ACKNOWLEDGMENTS

The assistance of Dr M. Prabhakaran for the preparation of the PEGylated hemoglobin models is greatly appreciated. This research has been supported by a grant-in-aid from the American Heart Association Heritage Affiliate, the National Institutes of Health grants HL-58427 and HL-71064, and the US Army grant PR023085.

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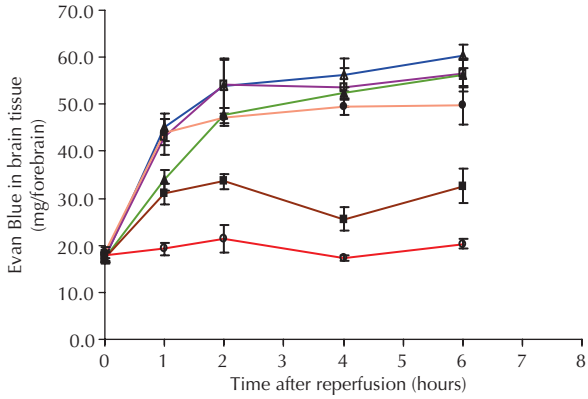

Plate 16 Effects of PolyHb–SOD–CAT on blood–brain barrier. PolyHb–SOD–CAT significantly attenuated the severity of blood–brain barrier disruption as compared to reperfusion with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb. $P < 0.01$. (From Powanda and Chang, 2002, with permission.) See Fig. 38.5.

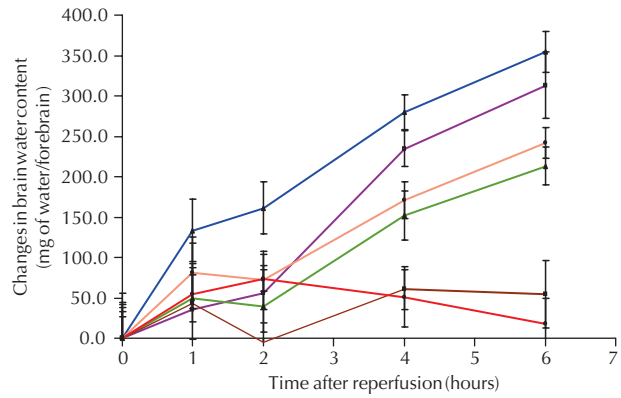

Plate 17 Brain edema: changes in brain water content. The changes in brain water content of PolyHb–SOD–CAT treated animals were not significantly different from that of the sham control. The increase in water content when reperfused with (1) oxygenated saline, (2) hemoglobin, (3) a solution of hemoglobin, catalase and superoxide dismutase and (4) PolyHb was significantly different from that of the sham control and PolyHb–SOD–CAT group by the fourth hour and increased there on with time. $P < 0.01$. (From Powanda and Chang, 2002, with permission. See Fig. 38.6.

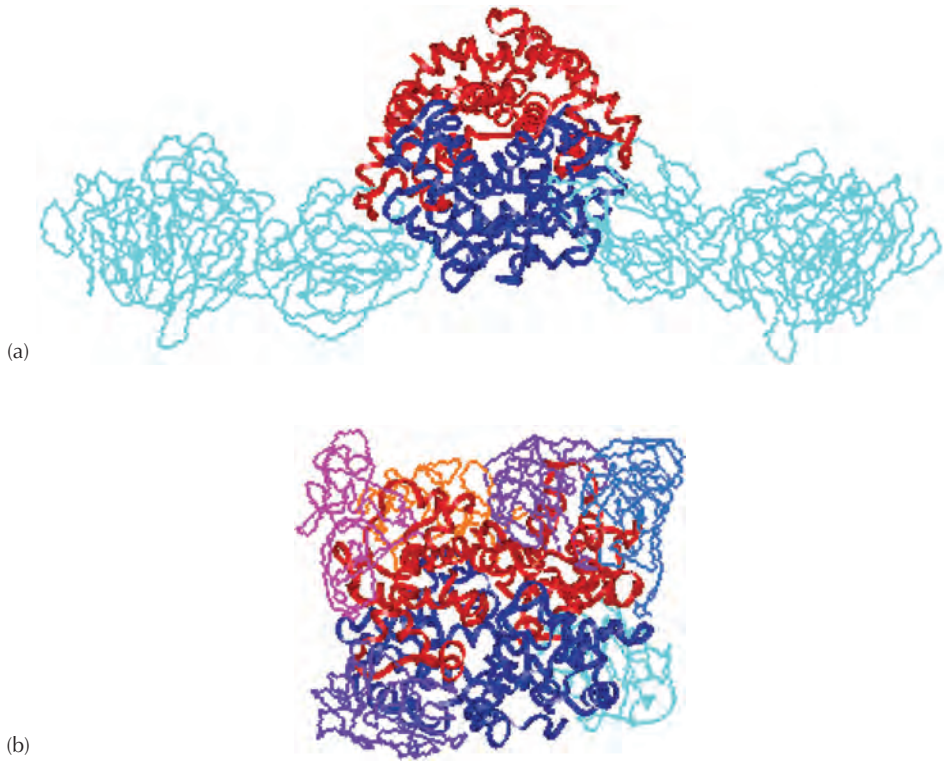

Plate 18 Molecular models of hemoglobin with two PEG 20 000 chains and six PEG 5000 chains. See Fig. 39.4.

Chapter 40

Hemospan® (MP4), A Human Hemoglobin Modified with Maleimide-Polyethylene Glycol

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INTRODUCTION

A safe and effective alternative to the infusion of allogeneic blood has been a long-held scientific and commercial goal. However, most hemoglobin-based products have suffered from problems that have prevented realization of their potential; the most recent and vexing is the propensity to cause hypertension and vasoconstriction. Development of MP4 began at the University of California, San Diego, where a study of modified hemoglobins with various properties was carried out in order to understand basic mechanisms of vasoconstriction and hypertension.

Hemoglobin-induced vasoconstriction has been most commonly attributed to the well-known propensity of hemoglobin to bind and scavenge nitric oxide (NO), a potent endothelium-derived vasodilator. However, a series of hemoglobins of differing molecular volumes had the same *in vitro* NO binding characteristics, yet very different effects on blood pressure in a rat model (Rohlfs *et al.*, 1998; Vandegriff *et al.*, 2004). Instead, we found that hypertension correlated with the rates of hemoglobin diffusion (McCarthy *et al.*, 2001). The well-known ability of hemoglobin and myoglobin to 'facilitate' oxygen diffusion (Wittenberg, 1970; Bouwer *et al.*, 1997; Nishide *et al.*, 1997), coupled with prior studies of autoregulation in the microcirculation (Lindbom *et al.*, 1988; Marini *et al.*, 1990) led to the hypothesis that hemoglobin-induced vasoconstriction can result from oversupply of oxygen to vascular walls (the autoregulatory theory; Winslow, 2003).

A consequence of the autoregulatory theory is that reducing hemoglobin diffusion by increasing its molecular radius and/or its viscosity and increasing its oxygen affinity would eliminate or reduce vasoconstriction (Vandegriff and Winslow, 1995). Surface modification of hemoglobin with polyethylene glycol (PEG) is an ideal way to accomplish this, since PEG is known to be nontoxic and is widely used in the pharmaceutical industry. The molecular properties of MalPEG-Hb and the formulation of MP4 have been optimized so that delivery of O_2 to vascular walls is the same as that of human red blood cells, as measured in an artificial capillary system (McCarthy *et al.*, 2001). Hemoglobin polymerization might also be effective, but the chemical reactions are difficult to control, expensive and provide relatively low yield, in our experience (Marini *et al.*, 1989, 1990).

THE AUTOREGULATORY HYPOTHESIS

The autoregulatory hypothesis is illustrated in Figure 40.1. From the lung, red cells are conducted through a cascade of vessels of progressively narrower diameter. Normally, the plasma

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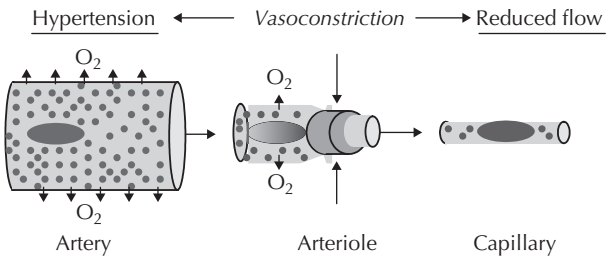

Figure 40.1 Effect of infusing an early-generation blood substitute with high diffusivity, low viscosity and low O_2 affinity. (From Winslow, 2004). See color plate 19.

space between the red cell and the vessel wall limits the diffusion of O_2, primarily because of its very low solubility in plasma. In the arteriole the distance for diffusion is less as vessels narrow, and terminal (precapillary) arterioles can adjust their diameter in response to the available O_2. This mechanism has been invoked to explain how O_2 supply can regulate capillary perfusion (Duling and Berne, 1970; Guyton, 1996).

We hypothesize that when cell-free hemoglobin with high diffusivity (small diameter, low viscosity) and low oxygen affinity is infused, diffusive transfer of O_2 to vessel walls is facilitated so that an excess of O_2 is detected in terminal arterioles, which respond by constriction and reduced capillary pressurization. The net result is two-fold: precapillary O_2 losses are increased, and the number of functioning capillaries is reduced. A further consequence, in many circumstances, is hypertension. Due to the limited diffusion, higher viscosity and high O_2 affinity, O_2 release by MP4 is limited so that the terminal arterioles do not detect increased O_2 supply. Thus functional capillary density is preserved, and precapillary O_2 losses are minimized. The small red dots in Figure 40.1 represent oxygenated plasma hemoglobin molecules. The blue dots represent deoxygenated plasma hemoglobin molecules. Excessive O_2 release in arterioles triggers vasoconstriction (arrows). The result is reduced flow and O_2 delivery to capillaries, and hypertension.

CHEMISTRY AND PHYSICAL PROPERTIES

Polyethylene glycol (PEG) itself is a non-toxic, amphiphilic polyether that is heavily hydrated in aqueous solution (Torchilin, 1997). Therefore when it is conjugated to hemoglobin, it greatly increases the molecular volume by virtue of a surface layer of water. Extensive studies have been done with PEG coating of liposomes (Zeisig *et al.*, 1996), showing that it prevents aggregation of red blood cells and binding of plasma proteins, including complement components, depending on the surface density and chain length of the PEG (Yoshioka, 1991). PEG has been used to modify a wide variety of proteins for human use (Burnham, 1994), including adenosine deaminase, asparaginase and Interferon, all of which are approved by the FDA. Still others are being developed, including superoxide dismutase (SOD), catalase, uricase, honey-bee venom and ragweed pollen extract.

Two PEG-hemoglobin conjugates have been previously developed as cell-free O_2 carriers (blood substitutes). These are pyridoxalated human hemoglobin polyoxyethylene (PHP) (Ajisaka and Iwashita, 1980) and PEG-bovine hemoglobin (Zalipsky *et al.*, 1991). The experience with these products has been helpful in evaluating the efficacy and potential toxicity of MP4, which differs significantly in that its oxygen affinity is higher, and it is more homogeneous. The specific PEG conjugation reaction used to produce MalPEG-Hb leads to an easily purified product, free of any side reactants or free hemoglobin.

Synthesis of MP4

The raw material for the hemoglobin component of MP4 is stroma-free hemoglobin (SFH) extracted from donor units obtained from licensed blood collection centers. They are therefore non-reactive for antibodies to HCV (hepatitis C), HIV hepatitis B core antigen, human T-cell lymphotropic virus, and West Nile virus. Units are negative for hepatitis B surface antigen, syphilis, hepatitis C (NAT) and HIV-1-RNA (NAT), and alanine aminotranferase (ALT) levels are within acceptable limits. All units are leukocyte-depleted. A further viracidal process (Goodrich, 2000) is used, in which the red blood cells are transferred to a special bag, mixed with riboflavin, diluted to a uniform hematocrit, and then illuminated in a special illuminator. The photoactivated riboflavin crosslinks nucleic acids, disrupting them and killing the viruses. Optimization and validation studies have identified energy levels of illumination which do not damage red blood cells, but which show robust reductions of bovine diarrhea virus (BVDV), pseudorabies virus (PRV) and HIV, and over 2 log

reduction of a small, non-enveloped virus, hepatitis A virus (HAV). The red cells are washed free of excess riboflavin and photoactivation products with saline.

The SFH is isolated by osmotic lysis, and then red cell membranes are removed by tangential flow filtration (TFF). SFH is passed through a 'VirA/Gard' (Ultrafiltration) 500-kD filter which has been validated for removal of viruses. SFH is concentrated by 10-kD TFF diafiltration with phosphate-buffered saline, passed through a 0.45-μm bioburden reduction filter, and then stored in flexible bags at −80°C.

MalPEG-Hb is prepared by thiolation of surface lysine residues with 2-iminothiolane (2-IT) and reacted with maleimide-activated PEG (MalPEG). MalPEG-Hb is purified using TFF to separate product from unreacted 2-IT, MalPEG, and hemoglobin. MP4 is formulated as MalPEG-Hb, 4.2 g/dl, in lactated Ringer's solution. MP4 is passed through a 0.45-μm bioburden reduction filter and then aseptically filled into glass bottles after filtration through two sequential sterilizing 0.22-μm filters. The final product is stored frozen at −20°C. Stability studies demonstrate that MP4 is stable for up to 15 months of storage at the standard storage condition of −20°C. MP4 remains within specification for methemoglobin (not more than 10 per cent) for approximately 3 months at 5°C.

Since no chromatographic methods are used in the preparation of MP4, some red cell enzymes are present in the final product. A chemical assay for the number of free sulfhydryl groups on the surface of hemoglobin yields two groups in native hemoglobin (the β93 cysteines), seven in thiolated hemoglobin, and one in finished product. Thus, the number of PEG chains is six per hemoglobin tetramer. Variation in molecular size of MalPEG-Hb is accounted for by the size range of the PEG, not the number of attachments.

Structure and physical properties of MalPEG-hemoglobin

The structural formula for MalPEG-Hb is shown in Figure 40.2. Since modification of hemoglobin with PEG increases its molecular size, gel permeation chromatography (GPC) is an effective way to monitor the reaction and the purity of the product. There is no detectable unreacted hemoglobin present in the final product (see Figure 40.3).

The symmetry of the MalPEG-Hb peak characterizes the homogeneity of the product. The optical spectrum of oxygenated MalPEG-Hb is similar

$$Hb-(S-Y-R-[O-CH_2-CH_2]_n-Y')_m$$

Figure 40.2 MalPEG-Hb formula. Hb refers to hemoglobin, S is a thiol group, Y is a succinimido group, R is a propyl group, Y′ is a methoxy group, n is the number of oxyethylene groups ($O–CH_2–CH_2$) per PEG polymer; m is the number of PEG polymers per Hb molecule.

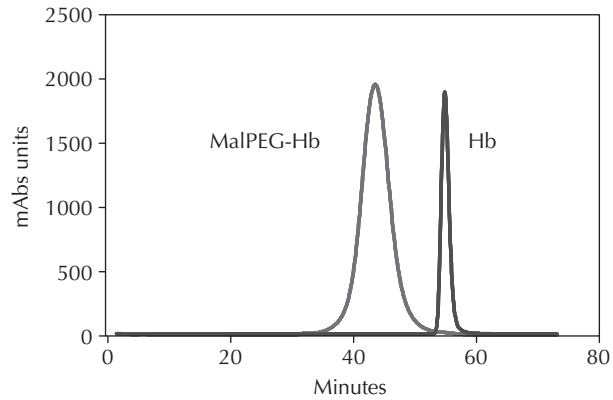

Figure 40.3 GPC pattern of MalPEG-Hb and unmodified human hemoglobin (Hb) (Vandegriff *et al.*, 2003).

Table 40.1 Properties of MP4 compared to blood and hemoglobin A_0

	Blood	Hb A_0	MP4
*P*50 (mmHg)	28	15	4–6
n (Hill coefficient)	2.9	2.9	1.2
Viscosity (cP)[1]	4.0	0.9	2.5
COP (mmHg)[2]	27	16	50
MW (kDa)	0	65	95
Molecular radius (nm)	4000	2.7[2]	10[2]

[1]Determined at 15 g/dl for whole blood and 4 g/dl for hemoglobin solutions.
[2]Determined by GPC and COP measurements (Vandegriff *et al.*, 1997).

to native human hemoglobin. Among the solution properties of MP4 (Table 40.1) are a *P*50 of 4–6 mmHg, and a radius of that is four times greater than that of native hemoglobin A_0. The relatively high viscosity and colloid osmotic pressure (COP) of MP4 compared to native hemoglobin are due to the strong interaction between polyethylene glycol chains and solvent water molecules. MalPEG-Hb can bind and release 1 molecule of O_2 for each heme group, hence, on a mole-for-mole basis, its O_2 capacity is the same as native human hemoglobin. Free iron is undetectable.

Functional properties of MP4

Early in the development of our theoretical conceptualization of the mechanism of vasoactivity induced by cell-free hemoglobin, we measured the NO reaction rate constant for all three classes of compounds and found them to be the same (Rohlfs *et al.*, 1998). Furthermore, the NO binding kinetics did not correlate with blood pressure measured in the rat when these solutions were infused. These kinetic NO binding experiments have been repeated using the current formulation of MP4 (Table 40.2). The rates of NO association to the deoxyhemoglobins and for NO oxidation of the oxyhemoglobins were identical for MalPEG-Hb and SFH.

The diffusive properties of representative modified hemoglobins were measured using a specially-constructed apparatus to simulate an arteriole (McCarthy *et al.*, 2001). The study compared $\alpha\alpha$-Hb, a PEG-modified bovine hemoglobin (PEG-BvHb), hemoglobin A_0, and a suspension of human red blood cells. The hemoglobin solutions were first equilibrated with air, then passed through the capillary and collected at the outlet to be analyzed for O_2 content. The gas outside of the capillary was pure N_2.

As the solution flows through the capillary, no O_2 is lost if the flow rate is high enough. If the flow is very slow, all the O_2 is lost, as the solution inside comes to equilibrium with the N_2 on the outside. At intermediate flow rates, the exit rate of O_2 depends on the diffusion of O_2 in the liquid, the oxygen affinity of the hemoglobin and the diffusion of hemoglobin itself ('facilitated diffusion'). The first two of these factors, O_2 diffusion and oxygen affinity, are known, and so differences in the behavior of the solutions that cannot be accounted for must be attributable to facilitated diffusion.

The results of the study showed that the overall transfer of O_2 by red blood cells was most nearly approximated by PEG-BvHb. In contrast, O_2 was delivered much more readily by $\alpha\alpha$-Hb compared to either red blood cells or PEG-BvHb. Oxygen release by hemoglobin A_0 was very similar to that of $\alpha\alpha$-Hb. These results can only be explained if the facilitated diffusion of the PEG-BvHb was attenuated compared to the smaller $\alpha\alpha$-Hb and hemoglobin A_0. The data support the concept that small hemoglobin molecules greatly increase the availability of O_2 to vessel walls.

Table 40.2 NO association (k′) and oxidation (k_{ox}) rate constants for stroma-free hemoglobin (SFH) and MalPEG-Hb (Vandegriff *et al.*, 2004)

	k′ ($\mu m^{-1}s^{-1}$)	k_{ox} ($\mu m^{-1}s^{-1}$)
SFH	21	18
MalPEG-Hb	22	23

NON-CLINICAL STUDIES

The classical hemodynamic response to the infusion of cell-free hemoglobin and some early hemoglobin based oxygen carriers (HBOCs) is the triad of elevated blood pressure, bradycardia and reduced cardiac output (Amberson *et al.*, 1949). Similar findings have been reported in more recent studies of crosslinked and polymerized hemoglobins (Winslow, 1992). This response was so extreme in a study of awake pigs, performed by the US Army (Hess *et al.*, 1993) using $\alpha\alpha$-crosslinked hemoglobin ($\alpha\alpha$-Hb), that this product was abandoned for further development. In order to be sure that MP4 would not result in similar findings, many preclinical studies were done in anticipation of clinical trials (Table 40.3).

Fifty per cent controlled hemorrhage and resuscitation in pigs

The circulation of the pig was noted to be a sensitive hemodynamic model in early US Army studies of $\alpha\alpha$-Hb (Hess *et al.*, 1991) and in subsequent studies of diaspirin crosslinked hemoglobin (DCLHb) performed by Baxter. Therefore we performed studies in pigs in collaboration with the Swedish Defense Establishment (FOI) in Söder Hospital, Stockholm, Sweden (Drobin *et al.*, 2004). The solutions studied included MP4 (4.2 per cent MalPEG-Hb in lactated Ringer's injection), stroma-free hemoglobin (SFH) and pentastarch (10 per cent Pentaspan®, B. Braun), and two mixtures of MalPEG-Hb and pentastarch (HS2, 2% MalPEG-Hb in 5% pentastarch, and HS4, 4% MalPEG-Hb in 5% pentastarch).

After insertion of pulmonary and systemic artery and central venous catheters for cardiovascular monitoring, animals were hemorrhaged 50 per cent of their blood volume followed by replacement of 70 per cent of the shed volume. Cardiac output and both systemic and pulmonary mean pressures remained higher than baseline levels. The fact that both flow and pressure rose in

Table 40.3 Summary of preclinical studies with MP4

Study	Species	Dose	Ref.
Hemodynamic response and oxygen transport after shock/resuscitation	Pig	35% of blood volume	Drobin *et al*. (2004)
Exchange transfusion followed by severe hemorrhage	Pig	40% of blood volume	Unpublished
Continuous exchange transfusion comparing MP4 and PEG-albumin	Rat	100% of blood volume	Winslow *et al*. (2004)
Effects of MP4 in an anesthetized model of hemorrhage and resuscitation	Rat	Hemorrhage to 25 mmHg; replacement with 50% of shed volume	Unpublished
Effects of MP4 in a model of uncontrolled hemorrhage	Pig	12 ml/kg	Young *et al*. (2005)
Targeted O_2 delivery by low *P*50 hemoglobin	Hamster	50% of blood volume	Tsai *et al*. (2003)
Blood kinetics of ^{14}C-labelled MP4	Rat	30% of blood volume	Unpublished
Tissue distribution of ^{14}C-labelled MP4	Rat	30% of blood volume	Unpublished
In vitro and *in vivo* oxidation	Rat, human	Various	Unpublished
Stability of MP4 after passing through a blood warmer	*In vitro*	N/A	Unpublished
Cytotoxicity and interference with viral infectivity assays	*In vitro*	N/A	Unpublished
Ames test on MP4	*In vitro*	N/A	Unpublished
MP4-induced interference with clinical chemistry measurements with human blood	*In vitro*	N/A	Unpublished
Chromosomal aberration on PET material	*In vitro*	N/A	Unpublished
Histological changes after acute infusion of MP4	Pig	50% of blood volume	Unpublished
15-day subacute toxicity/safety following transfusion with MP4	Rat	30% of blood volume	Unpublished
Cardiovascular safety during exchange transfusion	Rhesus monkey	30% of blood volume	Unpublished
Assessment of MP4 in a model of transient focal cerebral ischemia	Rat	10% top-load	Unpublished
Effect of MP4 on viability of cortical neurons in cell culture	*In vitro*	N/A	Unpublished

this circumstance suggest that resistance to flow was not appreciably affected.

Animals resuscitated with MP4 were able to maintain better oxygen uptake (VO_2) than the animals that received only pentastarch. The significance of this analysis is that in hemorrhagic shock, an oxygen debt is incurred – that is, oxygen uptake exceeds oxygen delivery, resulting in lactic acidosis if not corrected. Repayment of the oxygen debt is considered a necessary endpoint of resuscitation (Siegel *et al*., 1997).

From this series of experiments, we concluded that the effects of the three MalPEG-Hb formulations were not significantly different in terms of their hemodynamic effects. It was felt that the two formulations (MP4 and HS4) were preferred over HS2 because of the higher O_2 capacity, and that MP4 was preferred over HS4 because the interactions between the two entities, MalPEG-Hb and PS, have not been defined. Thus, MP4 was identified as Sangart's lead product for clinical trials.

Forty per cent hemorrhage and resuscitation followed by severe hemorrhage in pigs

A subsequent study in pigs, also performed at the FOI laboratory, compared the physiological effects of MP4, pentastarch and blood. Animals were first hemorrhaged by 40 per cent of estimated blood volume, and then resuscitated as before. After 1 hour, animals were subjected to a hemorrhage

that continued until death. This protocol allowed examination of the time-course of the metabolic changes that accompany hemorrhage as well as assessment of the overall length of time animals survive. Survival was in the order MP4 > pentastarch > blood. Base excess measured 1 hour after initiation of the second hemorrhage, a period at which all animals were still alive, showed that the animals given MP4 were in a more compensated acid–base state than the other animals.

Uncontrolled hemorrhage and resuscitation in pigs

This study was designed to model the use of MP4 in the setting of either pre-hospital trauma or acute, unexpected, severe intraoperative blood loss. Twenty-eight anesthetized pigs were hemorrhaged by catheter followed by rapid, uncontrolled hemorrhage from an aortic laceration. Total blood loss was approximately 45 per cent of estimated baseline blood volume. Following 15 minutes of uncontrolled bleeding, 250 ml of MP4, Ringer's acetate, stroma-free hemoglobin (SFH), or 10 per cent pentastarch were administered as resuscitation fluid, followed later by 250 ml of shed blood. Hemodynamics, acid–base status and blood gases were monitored for 20 hours following resuscitation. The principal results of the study were that six of seven MP4-treated pigs survived the duration of the protocol, compared to two of seven Ringer's acetate-treated pigs, two of seven SFH-treated pigs, and one of seven pentastarch-treated pigs.

MP4-treated animals demonstrated significant improvement of arterial blood pressure, cardiac output, O_2 delivery, pH control, base excess, lactic acid levels and urine output. These effects were not observed in the other three treatment groups.

Mean arterial pressure did not exceed baseline values in the MP4-treated animals; however, pulmonary artery pressure was higher in the MP4 and SFH-treated animals compared to Ringer's acetate-treated animals. Systemic vascular resistance fell in all groups except the SFH-treated animals, demonstrating the lack of systemic vasoconstriction with MP4. Pulmonary vascular resistance increased transiently, approximately two- to three-fold above baseline, in both the MP4 and Ringer's acetate-treated animals. The increase in pulmonary resistance was six-fold greater in the SFH-treated compared to MP4-treated animals, confirming that PEG conjugation effectively reduces the vasoactivity of hemoglobin.

Continuous exchange transfusion in rats

Since MP4 has a very low *P*50 (~6 mmHg), it was of interest to demonstrate that it delivers O_2 to tissue in addition to plasma expansion. To address this question, rats were continuously exchange-transfused with MP4, MalPEG-albumin or pentastarch (Winslow *et al.*, 2004). MalPEG-albumin was prepared using the maleimide-activated PEG and was formulated in lactated Ringer's solution at approximately 5 g/dl. This formulation, designated MPA, was matched with MP4 in terms of molecular size, oncotic pressure and the amount of MalPEG conjugated to its surface.

The exchange transfusion was carried out in awake rats for 60 minutes, which entailed exchange of approximately 2.5 blood volumes. Animals were then followed for an additional 70 minutes or until death. Surviving animals were sacrificed 130 minutes after start of the exchange. Both pentastarch and PEG-albumin animals died when the hematocrit reached approximately 5 per cent. In contrast, MP4 animals all survived the experiment, although with mild derangements of acid–base balance. No animals in the PS or MPA groups survived. Analysis of the 'critical hematocrit' (the hematocrit below which lactic acid accumulates) indicates that a plasma level of approximately 2 g/dl can support normal life in rats at a hematocrit of 5 per cent, while without plasma MP4 a minimal hematocrit of 15 per cent is required.

Microvascular response to hemodilution in Golden Syrian hamsters

The basic mechanism of action of MP4 is illustrated by experiments using the dorsal skinfold of the awake Golden Syrian hamster (Tsai *et al.*, 2003). This model allows direct visualization and measurement of arteriolar and venular width, blood flow and PO_2 in highly focused areas of the circulation. The solutions compared were MP4; polymerized bovine hemoglobin, 8-g/dl Oxyglobin™ (the commercial product available from Biopure Corp) and dextran-70. The oxygen equilibrium curves for MP4, Oxyglobin and normal hamster red blood cells are quite different from each other (Figure 40.4). The right-shifted Oxyglobin curve has a *P*50 of 53.4 mmHg, while the left-shifted MP4 curve has a *P*50 of 6 mmHg, compared to human blood, which has a *P*50 of about 29 mmHg.

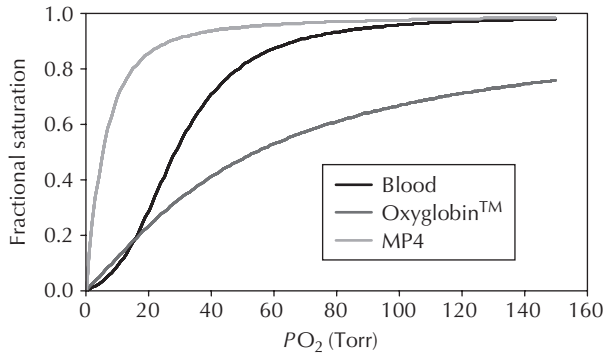

Figure 40.4 Oxygen equilibrium curves for MP4, hamster blood and Oxyglobin™ (Tsai *et al.*, 2003).

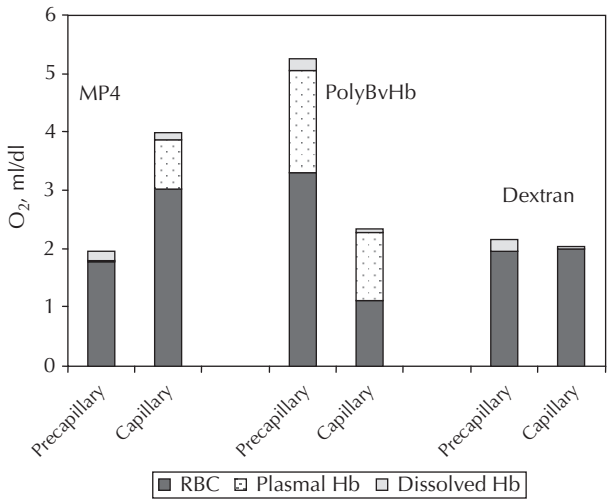

Figure 40.5 O_2 distribution in extreme hemodilution (Hct = 11 per cent) in the hamster (Tsai *et al.*, 2003).

This experimental model and the oxygen equilibrium curves shown in Figure 40.4 allow the calculation of the amount of oxygen released into precapillary vessels and capillaries (Figure 40.5). In the MP4 animals, 62 per cent of the total and 47 per cent of the red cell O_2 is released across the capillary beds, compared to 31 per cent of total and 14 per cent of red cell O_2 for the Oxyglobin animals and 40 per cent of total and 39 per cent of red cell O_2 for the dextran animals. This study concludes that not only does MP4 deliver significant O_2 to capillary beds at very low concentration and *P*50, but it also increases the efficiency of red cell O_2 delivery as well compared to the more conventional Oxyglobin. As an independent confirmation of this effect on O_2 delivery, the base excess values for the three groups of animals were 4.03 ± 1.68 mEq/l, −0.20 ± 1.68 mEq/l and −4.08 ± 3.52 mEq/l, respectively, for MP4, Oxyglobin and dextran animals.

The study also concluded that the functional capillary density (FCD) was significantly higher in the MP4 animals (63 per cent of baseline) compared to either the Oxyglobin (37 per cent of baseline) or dextran (36 per cent of baseline) animals. Thus, not only is more O_2 being delivered across individual capillary networks more efficiently, but also more networks are perfused in the MP4 animals compared to the other two groups of animals.

These parameters of microcirculatory O_2 delivery have also been measured in shock/ resuscitation experiments in hamsters (Wettstein *et al.*, 2003). After hemorrhage of 50 per cent of blood volume and resuscitation with an equal volume of shed blood, MP4, or hydroxyethyl starch, microvascular networks showed significantly better functional capillary density and microcirculatory blood flow in MP4 versus blood-resuscitated animals.

Hemorrhage/resuscitation in rats

In a comparison of MP4 with autologous blood, rats were hemorrhaged to a predetermined blood pressure of 25 mmHg and resuscitated with either lactated Ringer's solution, blood, or MP4 in a volume half of that removed. The post-resuscitation hematocrit in the MP4 animals was 16 per cent, and in the animals resuscitated with blood the hematocrit was 40 per cent. Nevertheless, recovery was faster in the MP4 animals and survival was equivalent, in spite of the low hematocrit and a plasma hemoglobin of only 1.8 g/dl in the MP4 animals.

Pharmacokinetics and metabolism in animals

The blood kinetics following a 30 per cent blood volume removal and intravenous infusion of ^{14}C-MalPEG-Hb (4 g/dl) were studied in the rat. Radiolabeled (^{14}C) 2-iminothiolane (2-IT) was prepared at New England Nuclear/Perkin Elmer. Blood samples (100 μl) were collected from the tail vein of each rat at intervals after dosing to determine radioactivity. This study concluded that the plasma $T_{1/2}$ in rats is approximately 24 hours, and that there is no apparent gender difference.

To determine tissue distribution in rats, radioactive MalPEG-Hb was administered as a 30 per cent blood volume exchange transfusion to two

groups of animals, each with five males and five females. One group was sacrificed at 24 hours, the other at 168 hours (7 days). Urine and feces were collected at intervals throughout the study. Tissues were harvested at necropsy, and radioactivity was determined.

More than 30 per cent of the total dose was excreted in the urine after 7 days. However, only 2.3 per cent of the total dose appeared in the urine during the first 4 hours after dosing. Although this value was small in both males and females, the greater amount excreted in this early period in females was statistically significant. In contrast to urine, less than 10 per cent of the administered dose appeared in the feces in the same period, and there appeared to be no difference between males and females. Radioactivity accumulated in the liver, kidney and spleen – organs that are known to be involved in the metabolism and excretion of hemoglobin.

In vivo oxidation

Oxidation of hemoglobin renders it unable to bind O_2, and the product, methemoglobin, has been implicated in certain toxicity issues (Alayash, 2000). Accordingly, two studies that model *in vivo* conditions were performed. In the first, MP4 was passed through a conventional clinical blood warmer in the manner in which it might be administered in an operating room. This process involves heating the liquid passing through the warmer to 37°C before entering a vein. The study found that the rate of oxidation in this model was unmeasurable.

The second study measured the methemoglobin formation in the rat after infusion. Awake, instrumented (arterial, venous catheters) animals were given a 50 per cent blood volume exchange transfusion and blood samples were collected for 48 hours. The results showed that after 24 hours the plasma total hemoglobin had declined to about 50 per cent of the injection concentration, but the methemoglobin level was still <10 per cent. Thus it appears that the conversion of oxy MP4 to met MP4 *in vivo* is not appreciable, most likely due the antioxidant properties of plasma.

TOXICOLOGY/SAFETY

Stroma-free hemoglobin, thiolated hemoglobin and MalPEG-Hb were tested at several dilutions in embryonic bovine trachea (eBTr) cells and in fetal rhesus lung (FRhL-4) cells. In both types of cell cultures, stroma-free hemoglobin and thiolated hemoglobin were cytotoxic in the undiluted form and at several log-fold dilutions. However, undiluted MalPEG-Hb (~3.5 g/dl) was not toxic in these cell lines. Conjugation of SFH with PEG clearly reduced the toxicity observed with SFH. Four reverse mutation studies were performed to evaluate whether MP4 would cause mutagenic changes in the number of revertants for the histidine-dependent *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, and in tryptophan-dependent *E. coli* strain WP2uvrA in the presence and absence of S9 metabolic activation. Several formulations of MP4 were tested, and all were non-mutagenic. A chromosomal aberration test in Chinese hamster ovary (CHO) cells confirmed that MalPEG-Hb is not genotoxic in this model.

Acute and subacute toxicology

Immediately after sacrifice in the hemorrhage/resuscitation pig study (Drobin *et al.*, 2004), approximately 2 hours after dosing, tissues were harvested, fixed and submitted to blinded histologic examination ($n = 8$ animals in each group). These studies showed no signs of abnormal exudates or transudates, no edema of the lungs or other organs, or any other significant findings. In samples of heart, ileum, kidneys, lung, pancreas, spleen, liver and skeletal muscle, no pathological lesions were found in any animal or in any organ that could be attributed to MP4.

The effects of transfusion with MP4 following 30 per cent blood volume removal was performed in 30 rats. The endpoints were survival, clinical pathology, clinical observations and histopathology, measured 14 days after dosing with MP4, pentastarch or lactated Ringer's solution. No significant differences were found in regard to body weight gain and food consumption. In all animals except those that received MP4, abnormalities were noted which included thinness, mild depression and rough hair/coat. There were no significant between-group differences with regard to clinical chemistry or hematology measurements. In particular, there were no significant changes in amylase or lipase. Minimal to moderate alveolar histiocytosis and mild to minimal renal tubular epithelial cell vacuolation were seen in the animals that received MP4. Animals that received pentastarch also demonstrated accumulation of vacuolated

macrophages in spleen and lymph nodes. The study concluded that infusion of MP4 is without adverse consequences.

Central nervous system toxicity

Concern has been raised in this regard because of unexpected negative results in a clinical trial of DCLHb in stroke patients (Saxena *et al*., 1998). In one study, the middle cerebral artery (MCA) was ligated in rats and reperfused after 2 hours with MP4 or lactated Ringer's solution. After euthanasia, no differences were found between the treatment groups in regard to infarct size, and neurological scores were significantly improved in the MP4 group at 24 hours compared to the 3-hour time point.

In a second study, MP4, lactated Ringer's solution, stroma-free hemoglobin (SFH) or whole blood was injected into the cisterna magna of anesthetized rats. MP4 had no effect on cerebral blood flow, but flow was reduced in the SFH and blood groups. Intracranial pressure rose transiently in all animals, but remained elevated only in blood-treated animals. SFH elevated mean arterial pressure – an effect not seen with MP4, blood or lactated Ringer's solution.

In a third study, cortical neurons from fetal mice were exposed to MP4 or SFH in culture. The results showed that release of lactate dehydrogenase (LDH) was significantly higher for the cultures exposed to MP4 compared to SFH. However, interpretation of these experiments is obscured because more metHb was present in the MP4 cultures compared to the SFH cultures. Since oxidation of MP4 is minimal *in vivo*, it is difficult to relate the results of this study to potential clinical toxicity.

Myocardial histology following 30 per cent blood volume exchange in primates

In November 2000, Baxter Healthcare disclosed that they had observed subendocardial necrosis in certain species, including swine (Burhop and Estep, 2001). Although the significance of this lesion was not known (Baxter proposed it was a result of NO scavenging), the FDA requested that Sangart perform the study in primates to establish whether MP4 caused the same lesion. Sixteen monkeys were studied (eight males and eight females). Animals received a single 30 per cent exchange transfusion with either MP4 or lactated Ringer's solution, then were observed for clinical signs, and changes in body weight, food consumption, behavior, electrocardiogram, serum chemistry, hematology, coagulation and urinalysis. Twelve animals were euthanized on Day 3 and the remaining animals were euthanized on Day 13.

No animal died as a result of administration of MP4, and no changes were noted in body weight, food consumption or behavior for animals that received MP4. No electrocardiographic abnormalities were attributable to the product. Significant rises were noted in serum lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), even after correction for spectrophotometric interference by the hemoglobin itself. There were no histopathological findings in skeletal muscle. There were no treatment-related effects on coagulation parameters (PT, aPTT, platelet count), and changes in urinalysis were limited to the presence of very small amounts of detectable hemoglobin on Day 3.

Gross necropsy did not reveal any alterations attributable to MP4 administration, at either Day 3 or 13, including organ weights. Microscopic changes were limited to the presence of foamy macrophages in the spleen and bone marrow, presumed due to phagocytosis of MP4 or a breakdown product.

In a separate study, 21 ml/kg of MP4 was administered to rhesus monkeys via exchange-transfusion at 5 m/min with continuous ECG monitoring. With the exception of one incident, all monkeys appeared electrocardiographically normal throughout the 7-day post-dose monitoring period. A single transient episode (approximately 5 seconds) of ventricular bigeminy in one animal was not considered to be related to treatment with MP4, and may have occurred in response to the exchange transfusion and/or stress during chair restraint. There were no alterations in blood pressure, heart rate or body temperature in any animals during or following infusion of MP4.

CLINICAL LABORATORY INTERFERENCE

Many clinical chemistry assays use spectrophotometric methods such that the presence of hemoglobin in plasma may interfere with correct measurement of certain analytes. Furthermore, MP4 is known to contain small amounts of normal red cell enzymes, which may be detected using conventional assay methods and

falsely elevate values obtained from clinical specimens. This problem is well known, and companies developing hemoglobin-based products have proposed correction procedures (Moreira *et al.*, 1997; Sarkozi *et al.*, 1997).

Studies were done on rhesus monkey serum to correct values from the GLP primate studies, and a similar protocol was used to determine the correction factors for human samples. Serum was collected from normal volunteers at the Karolinska Hospital and spiked with varying amounts of MP4 to achieve a range of serum hemoglobin concentrations encompassing those expected for anticipated human clinical trials (maximum $\leqslant 2$ g/dl). Samples from three volunteers were analyzed in duplicate on a Vitros 950 Chemistry System. This study concluded that no correction need be applied if the error between measured and predicted values is <10 per cent. For those assays where corrections were necessary, there is a good correlation ($R^2 > 0.8$) between the error and the hemoglobin concentration.

A separate study was performed to determine the interference of MP4 in clinical laboratory assays for Troponin T and Troponin I. Human plasma was spiked *in vitro* with a range of concentrations of Troponin in the presence or absence of MP4. The results of these studies demonstrated that there was little effect of MP4 on the measurement of Troponin I, and only a modest effect on the measurement of Troponin T.

A third study in rats found that MP4 does not interfere with oxygen saturation by pulse oximetry.

CLINICAL INDICATIONS

Preclinical animal studies have suggested three broad areas of potential clinical application for MP4. While the clinical situations differ, the potential application of MP4 in each of them results from MP4's ability to oxygenate tissue.

Hemodynamic stabilization

Presurgical administration, either by direct infusion ('top-load') or by partial exchange transfusion ('hemodilution') with consequent protection during severe hemorrhage suggests a role in maintaining hemodyamic stability during anesthesia induction and surgery. Hypotensive episodes during anesthesia induction, whether spinal or general, and during surgery, are a significant cause of postoperative morbidity. This morbidity is in the form of transient or permanent loss of psychomotor function, presumed due to temporary reduction in brain perfusion. Better maintenance of hemodynamic stability in surgery together with provision of oncotic pressure may also reduce the incidence of postoperative edema. All of these effects have the potential to reduce postoperative morbidity and to decrease the length of time for which patients are monitored in intensive care and, ultimately, their length of hospitalization.

Blood replacement after hemorrhage

Administration after severe blood loss, a traditional 'blood substitute' application, is supported by experiments in pigs and hamsters. In fact, MP4 has been designed to fill this role. MP4 could be administered intraoperatively after the first significant blood loss (the 'transfusion trigger') and during continuous hemorrhage in high blood-loss procedures. MP4 could also be given in the field to trauma victims, or on the battlefield, where blood is not available or in short supply.

Tissue oxygenation

The unique ability of MP4 to supply oxygen to hypoxic tissue while maintaining microvascular perfusion suggests that it might be effective in preventing or alleviating ischemia and/or hypoxia in specific tissues.

CLINICAL TRIALS

Phase I and II clinical trials have been conducted. At the time of writing, approximately 90 patients have been administered MP4, in doses up to 600 ml each. So far, no serious adverse events have been attributed to MP4.

A Phase I clinical trial (Bjorkholm *et al.*, 2005) was a First in Man study performed at the Karolinska Hospital and Institute in Stockholm, Sweden. Three dose levels were planned to be studied, 50 mg/kg, 100 mg/kg, and 250 mg/kg (approximately 100, 200 and 500 ml per patient, respectively), in three cohorts of four active and two control (placebo) normal volunteers. The third cohort (250 mg/kg) was not studied, as it was elected in consultation with the Swedish MPA to continue dose escalation in orthopedic surgery patients undergoing spinal anesthesia. In a Phase Ib/II study, 200, 400 or 600 ml of MP4

were infused into elective orthopedic surgical patients prior to spinal anesthesia induction. The primary objective of this study was to further document safety of MP4. Reversible elevations in some liver enzymes were noted in some patients, but these were not dose-related. Phase II studies in elective and emergent orthopedic surgery patients have been conducted in six Swedish medical centers. This was primarily a safety study, but exploration of efficacy in regard to hemodynamic stabilization was sought. In addition, the FDA has approved a Phase II study of intraoperative blood replacement in elective prostatectomy patients, and a third Phase II study has been proposed in sickle cell anemia patients.

SUMMARY

The clinical development plan for MP4 will focus primarily on its unique property of tissue oxygenation, and secondarily on volume expansion. Clinical trials in three broad areas are planned.

Animal studies have shown that pretreatment with MP4 protects animals from the effects of sudden, extreme hemorrhage. Accordingly, when MP4 was administered to elective orthopedic surgery patients, prior to or during spinal anesthesia induction, there was suggestive evidence that fewer hypotensive episodes and that greater fluid output in the immediate postoperative period might be expected. Despite optimal current therapy, clinical signs of hypovolemia have been reported in surgical patients undergoing spinal anesthesia.

MP4 was formulated as a balance between oxygen capacity and oncotic pressure, and with a concentration of 4.2 g/dl, it is not expected to be a complete replacement for blood. Continuous exchange transfusion experiments in rats, however, have shown that MP4 can permit reduction of the hematocrit by about 10 percentage points and maintain the same level of oxygen delivery to tissues as measured by lactic acid accumulation and base excess maintenance. It is expected that in most instances where blood transfusions are given, the remaining red cell mass would be the main source of oxygen. Oxygen transfer from red blood cells to tissue is facilitated by MP4. Many 1- or 2-unit transfusions are given in the immediate perioperative period, and it is believed that most, if not all, of these could be replaced by MP4 administration. As MP4 development continues, consideration will be given to the combination of presurgical treatment followed by further dosing either during surgery or postoperatively, as needed.

MP4 is perhaps most suited as an 'oxygen transport' agent, not strictly a blood substitute. As such, it represents an entirely new class of therapeutic agents.

EDITOR'S SUMMARY

MP4 (trade name Hemospan®) is a hemoglobin-based oxygen carrier (HBOC) consisting of 4.2 g/dl of chemically modified native human hemoglobin (MalPEG-Hb) in lactated Ringer's USP. The chemical modification has two main effects: reducing toxicity normally associated with high levels of plasma free hemoglobin, and ensuring that oxygen is transferred to maintain good capillary blood flow and tissue perfusion.

Preclinical animal studies have indicated that MP4 exhibits diminished hypertensive effects compared to unmodified, stroma-free hemoglobin. Based on animal studies, it is estimated that the therapeutic dose of MP4 will be in the range of 250–500 mg/kg body weight, or a maximum of about 1 l per patient. In rats, pigs and rhesus monkeys, MP4 is free of any significant toxicity in doses exceeding those proposed for human use. In particular, MP4 did not cause subendocardial microscopic lesions in rhesus monkeys.

MP4 is effective in low concentrations, in the range of 1–3 g/dl in the plasma. Biophysical studies, measurements of oxygen transport in an artificial capillary system, microcirculatory observations in the hamster, extreme hemodilution and shock studies in rats and pigs all suggest that MP4's reduced vasoactivity is due to the large molecular volume and high oxygen affinity, which limit engagement of vasoconstrictive mechanisms in arterioles. Furthermore, MP4 is oncotically active – a property that promotes volume expansion.

Phase I and II clinical trials have been performed in Europe. No serious adverse events have been attributable to MP4 administration to date. Potential clinical indications for MP4 include hemodynamic stabilization during anesthesia and surgery, blood replacement after hemorrhage, and treatment of ischemic tissue.

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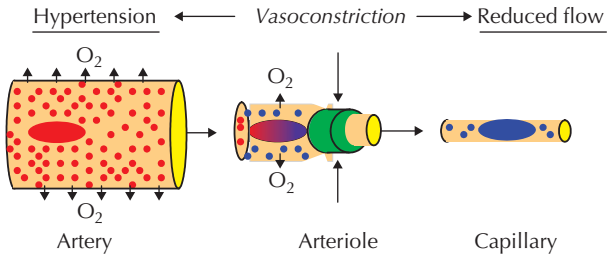

Plate 19 Effect of infusing an early-generation blood substitute with high diffusivity, low viscosity and low O_2 affinity. (From Winslow, 2004). See Fig. 40.1.

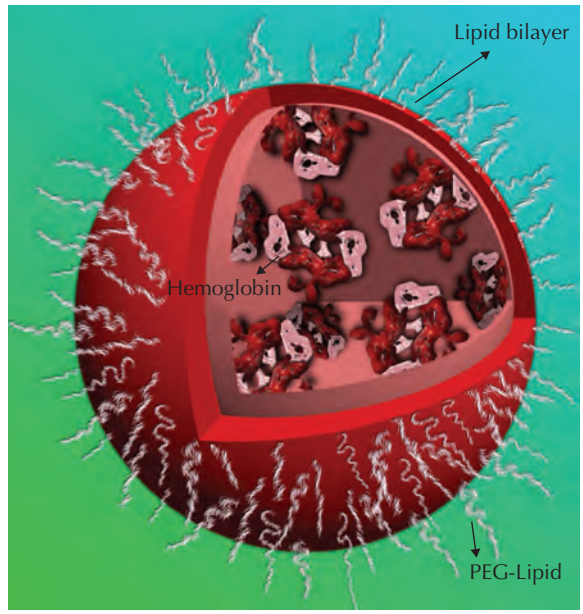

Plate 20 An illustration of liposome-encapsulated hemoglobin. See Fig. 43.1.

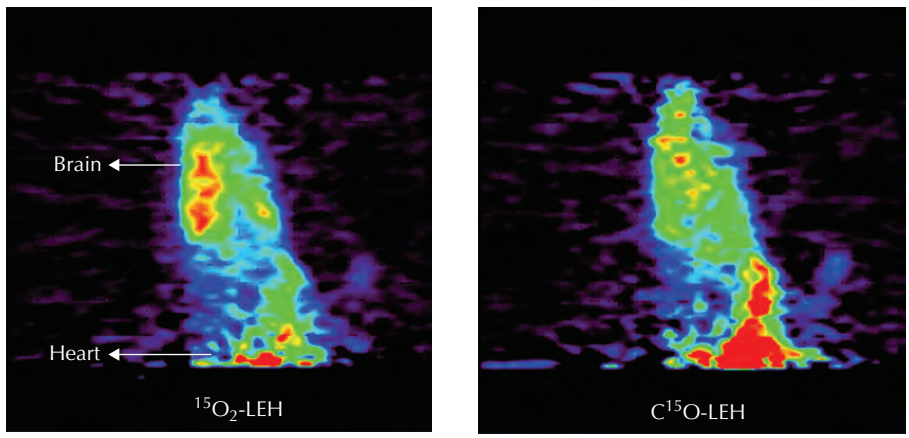

Plate 21 LEH was loaded with O_2 gas or CO (both labeled with O-15) and intravenously injected in a normal rat. Sagittal sections of PET images of rat brain are shown. The image acquisition was initiated with administration of labeled LEH and continued for 7 minutes. LEH releases O_2 to the brain tissue as indicated by the intensity of the brain image, but CO-labeled LEH predominantly shows blood volume in the brain. The degree of oxygen accumulation increases in the following order of color intensity: purple < blue < green < yellow < red. See Fig. 43.4.

Chapter 41

Dextran–Hemoglobin

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INTRODUCTION

In recent years the quest for a safe blood substitute has accelerated rapidly, propelled both by the hazardous nature of the blood supply in many parts of the world and by the increasingly satisfactory replacement of various plasma components with their recombinant counterparts. To replace the functions of erythrocytes and the bulk of plasma, a blood substitute needs to maintain effectively the oncotic pressure and volume of the circulation, and to carry oxygen from the lungs to support tissue respiration. Hemoglobin is an attractive oxygen carrier in the development of a clinical blood substitute, given its attributes as a respiratory pigment of extensive solubility, uptake and release of oxygen at appropriate partial pressures, buffering power, and above all its capability of transporting a large quantity of oxygen (Odling-Smee and Wilson, 1988). However, one fundamental disadvantage of free Hb itself as a blood substitute arises from its relatively small molecular size and consequent hemoglobinuria and rapid clearance from the circulation. In view of this, the development of hemoglobin-based oxygen carriers (HBOC) requires some method to slow down, or better still prevent completely, renal excretion of the Hb. A number of approaches have been utilized:

1. Crosslinking the two $\alpha\beta$-Hb dimers by chemical crosslinks in order to prevent dissociation of Hb into two halves
2. Achieving the same crosslinking by making recombinant α and/or β chains that are covalently joined together in such a way that the two $\alpha\beta$-dimers are linked and cannot dissociate
3. Polymerizing Hb to create high molecular weight polymers that cannot escape into the kidney tubules
4. Joining Hb to a polymeric carrier such that the polymer-Hb conjugate is too large to escape into the kidney tubules
5. Placing the molecules into liposomal sacs resembling red blood cells, so that Hb does not come out into the plasma and enter the urine.

These approaches have succeeded to various extents in overcoming the problem of urinary excretion and short plasma half-life caused by the small size of the Hb molecule. At first it was thought that once this problem had been resolved, a satisfactory HBOC would be readily

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obtainable. Experience over the past decade, however, has made clear that the design of a satisfactory HBOC will require maximizing the performance of all molecular aspects of the HBOC. In this regard, the covalent dextran–hemoglobin (DxHb) conjugate offers important advantages.

HIGH YIELD OF DxHB

DxHb is prepared by conjugating human Hb to dextran, a poly1→6-α-D-glucose containing randomly distributed 1→3-α-glucopyranosyl branch residues (Yalpani, 1988). Since dextran has long been used as a clinical plasma volume expander, its biocompatibility is well established. Its selection as carrier polymer in an HBOC is further suggested by its beneficial action on blood flow. The permeability from plasma to lymph of dextrans of molecular weight 8–500 kDa decreases with molecular weight (Muranishi, 1991), and dextrans of less than 51.3 kDa molecular weight are not immunogenic in man (Kabat and Bezer, 1958). Dextran is completely metabolized or excreted from the body after brief storage in the cells of the reticuloendothelial system, and it can be chemically modified by a variety of methods to form defined and stable compounds. Indeed, the combination of water solubility, availability in a wide range of molecular sizes, and lack of significant toxicity or tissue tropism renders dextran an excellent drug carrier among biodegradable polymers. Dextran conjugation has been employed to prolong the plasma half-lives of asparaginase (Wileman *et al.*, 1986), carboxypeptidase (Melton *et al.*, 1987), adenosine deaminase (Rosemeyer *et al.*, 1982) and arginase (Sherwood *et al.*, 1977). The coupling of proteins to dextran is therefore an important means to enhance their therapeutic efficacies.

DxHb is made by coupling Hb to bromo-Dx in a high-yield conjugation (Xue and Wong, 1994). Since the coupling is conducted under room air without any need for cumbersome deoxygenation, it may be readily prepared on a large scale.

PROTECTION OF KIDNEYS

The conjugation of Hb to dextran prevents both excretion of Hb and kidney damage. When DxHb synthesized from three different sizes of Dx (molecular weights 20, 40 and 70 kDa) are infused into rabbits, they do not enter into the kidneys, and their clearance rates do not vary with the size of the Dx moiety over this range (Blumentsein *et al.*, 1978). The non-excretion is not the result of interference of renal function by DxHb, because inulin excretion remains unimpaired in its presence.

In the rat model, infusion of stroma-free Hb causes a marked decrease in glomerular filtration rate, and an extensive elevation in urinary N-acetyl-β-D-glucosaminidase (NAG) activity as an indicator of structural damage to renal tubular cells caused by the passage of Hb into the tubules. In contrast, DxHb, because of its larger molecular size, does not pass through the glomeruli into the tubules, and leads only to a minor appearance in urinary NAG with no significant impairment in glomerular flltration rate (Tam and Wong, 1988).

NON-ENTRY INTO LYMPH

Importantly, conjugation to dextran also retards the non-renal clearance of hemoglobin. When free Hb is infused into the body, it is cleared from the circulation with a $t_{1/2}$ of only 45 minutes, but despite the massive hemoglobinuria, less than 40 per cent of the Hb is cleared via the renal route (Tam *et al.*, 1976). Therefore, a rapid non-renal clearance of Hb occurs. Non-renal escape of Hb into extravascular space is of utmost clinical significance, for it may aggravate tissue edema during shock and other pathological states. It is noteworthy in this regard that different HBOCs, albeit renally non-excretable, vary in their circulatory residence time as a result of their different rates of non-renal clearance. It is suggested that a long plasma residence time is an essential requirement for a satisfactory HBOC in order to provide assurance that non-renal extravasation compounding tissue edema would be low. Long residence time also minimizes the need to top up the recipient frequently to maintain plasma Hb level, limits tissue damage induced by iron overload, and cuts down on the cost of HBOC treatment. Because DxHb does not enter the lymph (Tsai and Wong, 1997), its physical plasma half-life is 58 hours in dogs (Tam *et al.*, 1978) – one of the longest among different forms of HBOC. The fact that DxHb can function as an HBOC containing only 6 per cent conjugated Hb further reduces both iron overload and costs.

OXYGEN AFFINITY

DxHb binds and releases oxygen reversibly with an affinity that is 2.5-fold higher than that of Hb, with an *in vivo* half-saturating oxygen partial pressure (*P*50) of 7 mmHg (Tam *et al.*, 1976). The oxygen affinity can be decreased by the covalent attachment of the right-shifting agent oxidized inositol tetrakisphosphate (oxyIP4). The oxyIP4 is readily obtained from partial phytase digestion of inositol hexakisphosphate, or phytate, followed by periodate oxidation to yield the dialdehyde. Covalent attachment of the dialdehyde to DxHb is achieved through reductive alkylation with dimethylamine borane as reducing agent (Wong, 1988). Since the attachment effectively abolishes further right-shifting response of the Hb molecule to phytate, oxyIP4 evidently is attached to the same polyphosphate site as phytate (Xue *et al.*, 1992). The *P*50 of oxyIP4 right-shifted DxHb is 23 mmHg. Because of the reduced Hill coefficient of DxHb relative to erythrocytes, during oxygen unloading from 100 per cent down to 50 per cent saturation, which is the normal operating range of unloading *in vivo*, the oxygen dissociation curve for right-shifted DxHb will be slightly right-shifted relative to the erythrocyte curve over much of this range. By controlling the percentile oxyIP4 modification, the *P*50 of DxHb may be varied continuously between 7 and 23 mmHg to fit optimally the application of the DxHb. Thus a low oxygen affinity may be expected to expedite oxygen release by the HBOC to the tissues, and a high oxygen affinity may be expected to reduce venous oxygen tension and potential free radical toxicity of the HBOC (Alayash, 2000).

PHYSICAL STABILITY

Covalent coupling of Hb to dextran reduces its affinity for haptoglobin (Tam and Wong, 1980). It also enhances the stability of Hb against acid denaturation and ethanol precipitation. Unlike free Hb, which begins to precipitate when ethanol concentration exceeds 10 per cent, DxHb only begins to precipitate at 30 per cent ethanol. This opens the way to possible sterilization of DxHb solutions with organic solvents. The enhanced physical stability of DxHb provides an extra safeguard against the precipitation and denaturation of the hemoglobin in the bloodstream causing circulatory obstruction. That the autoxidation of hemoglobin is slowed by 30 per cent when complexed to dextran (Wong, 1988) also enhances the utility of DxHb as HBOC.

IN VIVO STUDIES

Exchange transfusion

When dogs were exchange transfused with either 10 per cent dextran or 6 per cent DxHb (with respect to Hb moiety) in kidney dialysis fluid, the animals exchange transfused with dextran could not survive when hematocrit was reduced to the 6–10 per cent range because of lack of oxygen-delivering capacity. In contrast, the hematocrit could be lowered to 2 per cent or less in the DxHb exchange-transfused dogs, whereby the animals depended entirely on oxygen delivery by DxHb for life support. The infused DxHb exhibited a physical plasma $t_{1/2}$ of 58 hours and a functional plasma $t_{1/2}$, after correcting for non-functional Dx-metHb, of 46 hours. Because of the prolonged functional half-life of DxHb in circulation, and the rapid erythropoiesis following the blood replacement, the animals went on to complete recovery under room air without any need for further transfusion. The rapid erythropoiesis observed indicates that the bone marrow was being supported vigorously by DxHb following the blood replacement. Since the bone marrow is one of the most fragile tissues in the body, with its populations of rapidly dividing cells being particularly susceptible to injury by radiation and chemicals, the health of this tissue constitutes strong evidence of the ability of DxHb to support cell proliferation and function.

Hemorrhagic shock

While the exchange transfusions carried out with dogs demonstrated the capacity of 6 per cent DxHb to support life at less than 2 per cent hematocrit, it is important also to examine its ability to do so under conditions of hemorrhagic shock. Accordingly, guinea pigs were subjected to hemorrhagic shock and resuscitated with DxHb. The results showed that DxHb was capable of maintaining life under both exchange-transfusion and hemorrhagic shock conditions (Table 41.1).

POTENTIAL FOR DxHb

Dextran–hemoglobin is the first soluble blood substitute to sustain, without any need for further transfusion, complete recovery under room air of

Table 41.1 Resuscitation with DxHb of guinea pigs in hemorrhage shock. Animals were anesthetized with pentobarbital ip and bled to a 70 per cent of blood volume over 10 minutes. The blood pressure dropped from 85 to 30 mmHg. Subsequently, 0.2–0.5 ml was bled occasionally for the following 80 minutes to keep the blood pressure at 25–30 mmHg. At 90 minutes, a bled volume of resuscitating fluid was infused over 60 minutes. Only those animals with a 90-minute lactate level between 50 and 90 mg/dl were included in the tests (Tsai and Wong, unpublished data)

Resuscitation treatment	N	Survival (per cent)
Kidney dialysis fluid (KDF)	7	75
5 per cent Hb in KDF	6	50
5 per cent DxHb 70–500 kDa in KDF*	9	100

*Obtained with $<$500-kDa A/G filter cartridge.

animals that have undergone essentially complete replacement of erythrocytes. This is made possible by its relatively long residence time in plasma, such that its disappearance from the bloodstream is adequately compensated for by the vigorous erythropoiesis it supports. The long residence time is in turn the result of non-clearance through the renal route, which protects the integrity of kidney function and structure, as well as slow clearance through non-renal routes including extravasation and cellular uptake processes. Slow extravasation would usefully limit tissue edema. The long residence time also reduces the frequency of HBOC infusions required to maintain an adequate hemoglobin level in blood. This reduction together with the relatively low concentration (6 per cent) of Hb-moiety required by a DxHb HBOC achieves economy with regard to both the metabolic iron load imposed on the recipient and the cost of the HBOC, which is expected to be a critical factor determining the usefulness of the HBOC in many countries of the world.

EDITOR'S SUMMARY

Coupling of hemoglobin to a larger molecule, in this case dextran, greatly increases its molecular size. This technology was developed prior to crosslinking with DBBF, but was not initially developed as thoroughly. Since the lessons learned from $\alpha\alpha$-Hb and DCLHb have been fully appreciated, attention has turned to ways of increasing the size of the cell-free hemoglobin molecule in order to reduce its vasoactivity and extravasation. DxHb has a number of promising features, including extended plasma retention ($T_{1/2}$), apparently reduced vasoactivity and ease of production. In contrast to hemoglobin conjugated to PEG, DxHb has less oncotic activity, potentially allowing higher hemoglobin concentrations.

However, dextran is not as commonly used today, at least in the US, because of the significant incidence of hypersensitivity reactions and potential for coagulation defects. Hence, the chief contribution of this research may be in pointing to conjugation with other polymers.

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Chapter 42

Development of Non-Extravasating Hemoglobin-Based Oxygen Carriers

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INTRODUCTION

Three main problems affect the development of physiologically competent cell-free, hemoglobin-based oxygen carriers (HBOC). First, the loss of 2,3 BPG upon hemolysis of the red cells implies an increasing oxygen affinity of purified hemoglobin to *P*50 values near 12 mmHg, an affinity much higher than that of blood with *P*50 near 30 mmHg, which was considered too high for an efficient oxygen transport. Second, purified hemoglobin allows its dimeric species to filter through the endothelium of the glomeruli and other capillary beds, producing a profuse hemoglobinuria and flooding of internal organs. Extravasation also contributed to shortening of the half-time of intravascular retention to 30–45 minutes in the rat (Urbaitis *et al.*, 1991). A third important problem is the systemic vasoconstriction that follows the infusion of cell-free hemoglobins, with consequent increase in mean arterial pressure (MAP).

Supported by federal funds, at the University of Maryland Medical and Dental Schools and at Johns Hopkins Medical Institutions in Baltimore, we attempted a systematic investigation of these problems. We used chemical modification of hemoglobin molecules (either human or bovine) to obtain systems with longer retention time and 'low' oxygen affinity (i.e., comparable to that of blood, and possibly lower) and without production of hemoglobinuria. Employing a technology originally developed by Klotz (Zaugg *et al.*, 1980), 3,5-dibromosalicylic acid-activated aliphatic dicarbocylic acids were used to insert a covalent bridge between the two β82 Lysines of the partner β subunits in the β-cleft of human hemoglobin (or the β81 Lysines of bovine hemoglobin). The prevention of dimer formation should have decreased the hemoglobinuria and prolonged the retention time.

FUMARYL-CROSSLINKED BOVINE HEMOGLOBIN

Fumaryl-crosslinked bovine hemoglobin (DBBF-BvHb) had a *P*50 near 17 mmHg and a low

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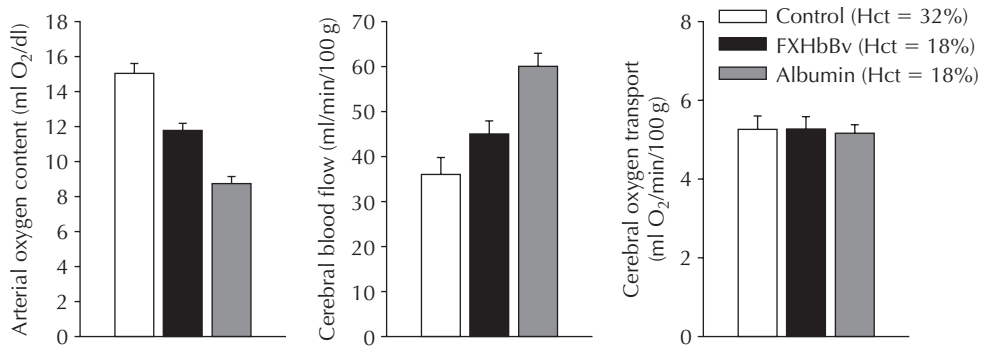

Figure 42.1 Arterial O_2 content, cerebral blood flow, and cerebral O_2 transport (blood flow × arterial O_2 content) in a time control group and groups of anesthetized cats exchange-transfused approximately 40 per cent of blood volume with fumaryl crosslinked bovine hemoglobin (DBBF-BvHb) and albumin. The increase in cerebral blood flow after exchange-transfusion compensated for the decrease in arterial O_2 content and kept cerebral O_2 transport to the microcirculatory exchange site unchanged. Values are mean ± SE (adapted from Ulatowski *et al.*, 1996).

oxygen-binding cooperativity with a Hill's parameter (n) near 1.2. In this preparation, there were residual non-crosslinked species, dissociable into dimers, which resulted in extravasation and modest hemoglobinuria. DBBF-BvHb infusion produced a 10–20 per cent increase in MAP (Ulatowski *et al.*, 1996). As expected, the half-time of intravascular retention time was prolonged to near 3.5 h in the rat (Urbaitis *et al.*, 1991). The prolonged retention time allowed us to observe that, in 40 per cent exchange-transfused cats, infusion of the carrier restored cerebral blood flow during anemia to near normal levels and preserved bulk oxygen transport to the brain (Ulatowski *et al.*, 1996a) (Figure 42.1). Differences in cerebral blood flow between exchange transfusion of either albumin or DBBF-BvHb persisted after nitric oxide synthase (NOS) inhibition, suggesting that differences in blood flow were largely attributable to differences in oxygen-carrying capacity rather than nitric oxide (NO) scavenging. These findings implied that this carrier delivered oxygen to tissue in spite of the high affinity and low cooperativity. Notably, the restored amount of cerebral blood flow was obtained with a plasma concentration of DBBF-BvHb near 1.5 g/dl, less than half that of the red cell hemoglobin lost with the exchange transfusion, which was near 4 g/dl. That observation raised the possibility that oxygen delivery of the cell-free carrier was more efficient than that of the red cells.

In view of the MAP increase, using microsphere technology we measured changes in the blood flow in various organs that were produced by infusion of the cell-free carrier. As a consequence of the vasoconstriction that increased MAP, blood flow decreased unevenly among the various organs. The vasoconstriction was greatest in the intestine, kidney and pituitary (Ulatowski *et al.*, 1996b). Importantly, identical decreases could be reproduced in control animals treated with NOS inhibitors (Matheson *et al.*, 1998; Ulatowski *et al.*, 1996b). These data suggested that NO scavenging by cell-free carriers plays a major role in producing a systemic vasoconstriction, which can adversely affect vital organs. The contrast in the blood-flow response between the pituitary, which does not have a blood–brain barrier, and the rest of the brain suggested that organs without tight endothelial junctions are more prone to the vasoconstrictive effects of cell-free hemoglobin transfusion.

SEBACYL-CROSSLINKED TETRAMERIC HEMOGLOBIN (DECA)

Continuing our studies, we observed that by using activated dicarboxylic acids of increasing length, the covalent bridges in the β-cleft of hemoglobin, besides preventing dimer formation, decreased the oxygen affinity by an amount directly proportional to the length of the bridge. Fourteen-carbon-long bridges could be inserted into the β-cleft of human hemoglobin. However, bovine hemoglobin did not form covalent bridges longer than six carbon atoms. Longer chains failed to go 'across' the cleft with proper orientation. Each of the two β81 Lysines in the

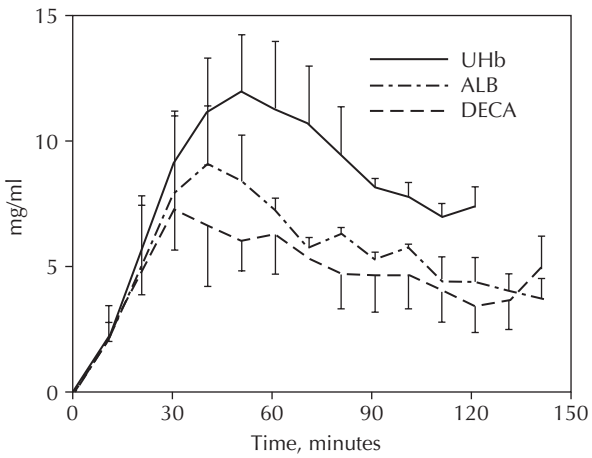

Figure 42.2 Transients of hilar lymph concentration in rats 30 per cent exchange-transfused with unmodified hemoglobin (UMB), DECA and marked albumin (adapted from Matheson *et al.*, 2000).

partner subunits were separately substituted with the reagent (unpublished).

We focused on the use of DECA, i.e., human hemoglobin crosslinked with sebacic acid (10 carbons long) (Bucci *et al.*, 1996). Crystallographic analysis showed that in deoxy-DECA, the sebacyl bridge was extended between the two β82 Lysines without disturbing the structure of normal deoxygenated human hemoglobin. However, computer modeling suggested that the structure of human oxy-hemoglobin could not accept the insertion of the sebacyl residue without distortions of the inserted bridge and repositioning of the β82 Lysines and some nearby amino acid side chains. This distortion of the crosslinked β_1–β_2 interface of the liganded structure was probably the cause of the reduced oxygen affinity of DECA (*P*50 near 30 mmHg). This compound also conserved a cooperativity with a Hill's parameter (n) near 2.0. These preparations contained a 10 per cent residual of non-crosslinked hemoglobins, which was eliminated by affinity chromatography.

In the absence of dissociable material, these stabilized tetramers did not produce hemoglobinuria. However, the half-time of intravascular retention was still near 3.5 h in the rat, much lower than the half-time for marked serum albumin of similar size, and very similar to that for DBBF-BvHb. DECA also produced MAP increase in the cat similarly to DBBF-BvHb. A new finding was that DECA, while not appearing in the urine, appeared in the hilar lymph of the rat kidneys. Thus, this crosslinked tetramer was retained by the endothelial walls of the glomeruli, but not by the large pores of other capillary beds (Matheson *et al.*, 2000). This was an important and unique observation, which revealed that extravasation was concomitant with MAP response in infused animals (Figure 42.2) and explained the still short intravascular retention time.

Through a cranial window in 40 per cent exchange-transfused cats, we measured the increase in diameter of brain arterioles upon external addition of acetylcholine and ADP. These vasodilation responses, which depend on NO released by the endothelium, were not affected by the presence in plasma of about 1.5 g/dl of infused DECA. In contrast, the vasodilation was inhibited when DECA was added on the surface of the brain, i.e., on the abluminal side of the arterioles, starting at concentrations at least three orders of magnitude less than the plasma concentration (Asano *et al.*, 1998; Figure 42.3). Therefore, the amount of scavenging of NO in the luminal side of the vessels by DECA was insufficient to impair this endothelial-dependent response. Moreover, these data predict that only a small fraction of transfused hemoglobin is required to extravasate in order to interfere with NO-dependent dilation. In the brain with tight endothelial junctions, extravasation of hemoglobin is inadequate to compromise the acetylcholine and ADP responses. These findings imply that when a plasma-based hemoglobin interferes with an NO-dependent response, the interference is more likely the result of extravasation rather than scavenging of NO in the luminal side of the arterials. As discussed below, a large polymeric hemoglobin, which does not extravasate into renal lymph and does not produce hypertension, also does not interfere with acetylcholine-evoked dilation of pial arterioles (Rebel *et al.*, 2003). Consequently, the increase in MAP observed with crosslinked tetrameric hemoglobin is probably related primarily to extravasation of tetramers through the large pores of peripheral vascular beds (Rippe and Haraldsson, 1994).

It is important to know whether transfusion of cell-free hemoglobins interferes with the ability of the vasculature to respond to other physiological stimuli, such as hypoxic hypoxia. Increases in cerebral blood flow were noted when inspired oxygen was reduced to different levels in anesthetized cats after transfusion of DECA (Ulatowski *et al.*, 1998a). The magnitude of the increase in blood flow was found to be

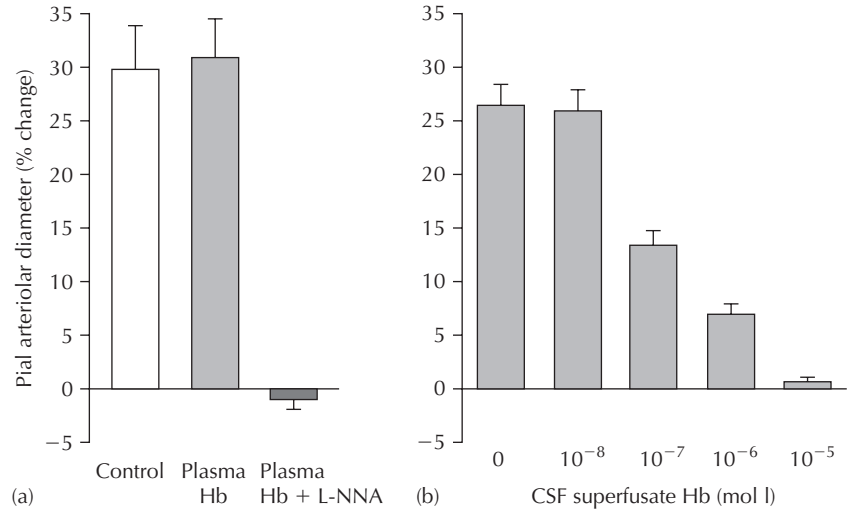

Figure 42.3 Percentage change (± SE) in pial arteriolar diameter during superfusion of 30 μM acetylcholine in anesthetized cats with a closed cranial window. (a) Response in non-transfused control group and a group exchange-transfused with DECA Hb (plasma Hb concentration of ~0.5 mmol/l) and with responses measured before and after application of the NO synthase inhibitor N^w-nitro-L-arginine (L-NNA; 300 μM). (b) Response to acetylcholine when CSF in window over the arterioles was superfused with increasing concentrations of DECA Hb. Note that abluminal DECA began to inhibit acetylcholine dilation at submicromolar concentrations, whereas intraluminal DECA at a plasma concentration over three orders of magnitude greater had no effect on the vasodilatory response, which was dependent on nitric oxide synthase (NOS) activity (adapted from Asano *et al.*, 1998).

appropriate for the level of arterial oxygen content; i.e., at a particular level of arterial oxygen content, the cerebral blood flow was the same as that found in control cats that did not undergo a transfusion. Moreover, the increase in cerebral blood flow during hypoxia maintained bulk cerebral oxygen transport (arterial oxygen content × cerebral blood flow) at the same level observed during normoxia (Figure 42.4). Similar results were obtained for coronary blood flow. Therefore, the presence of a significant amount of stabilized hemoglobin in the plasma does not interfere with the normal blood flow responses to hypoxic hypoxia in the brain and heart. The intact vasodilatory response to hypoxic hypoxia in the presence in plasma of 1.5 mg/ml DECA implies that vasodilation is not produced by NO released from nitrosylated red cell hemoglobin, as proposed by Stamler and colleagues (McMahon *et al.*, 2002), because released NO would be readily scavenged by heme in the plasma.

We tested the efficiency of oxygen delivery by DECA in maintaining the functional viability of intestinal membranes perfused in Ussing chambers (Bucci *et al.*, 2003). When rabbit jejunal membranes are voltage-clamped between the two sides of Ussing chambers and are perfused with Ringer's solutions, transport of amino acids and other metabolites from the mucosa to the serosa side of the membrane generates an electric current, the amperage of which is directly proportional to the amount of transported metabolite. Classically, perfusates are equilibrated with 95% oxygen and 5% CO_2 to allow functional survival of the membrane for at least 4 h. Upon addition of 3 g/dl DECA in Ringer's perfusate, the equilibrating oxygen fraction could be lowered to 30 per cent. At this lower oxygen pressure, more similar to that in a normal atmosphere, a 3-g/dl hemoglobin-equivalent suspension of bovine red cells was not able to support metabolite transport, and the tissue was non-viable within 30 minutes (Figure 42.5). Notably, the oxygen-binding characteristics of DECA are similar to those of a bovine red cell suspension, with a *P*50 near 30 mmHg and a Hill's parameter (n) near 2.7. The oxygen bound by the red cells was not readily available for metabolic consumption; its emergence from the red cells was retarded by the internal viscosity of cytoplasm, by the cell membrane, and by the diffusion through the

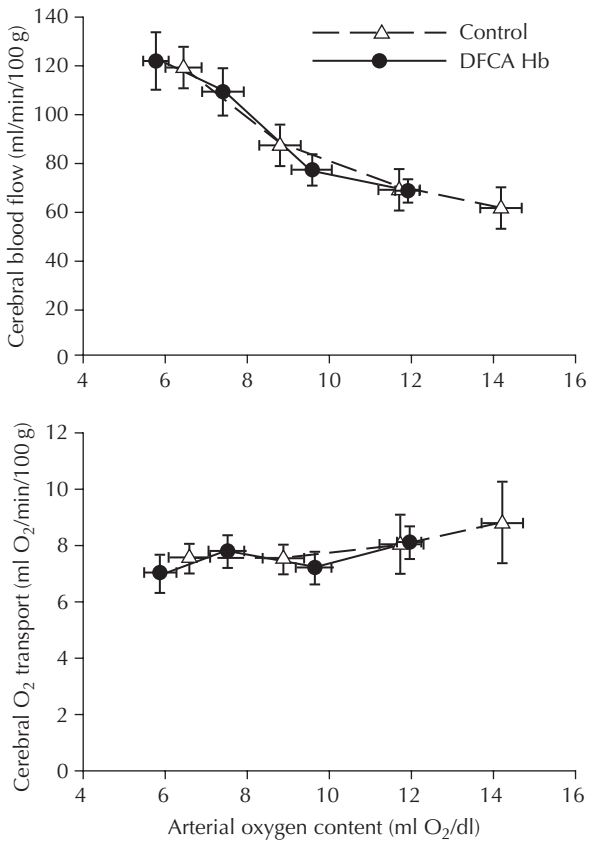

Figure 42.4 Cerebral blood flow and cerebral O_2 transport (blood flow × arterial O_2 content) in anesthetized cats during graded decreases in arterial O_2 content produced by decreasing inspired O_2 in non-transfused controls and after exchange transfusion of DECA Hb. Values are mean ± SE (adapted from Ulatowski *et al.*, 1998).

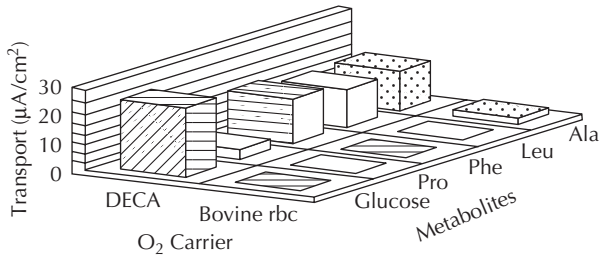

Figure 42.5 Transport of glucose and amino acids through a rabbit jejunal membrane voltage clamped in a Ussing chamber. The Ringer's perfusate was equilibrated with 30% O_2, 5% CO_2, and contained either 3 g/dl DECA or an equivalent suspension of bovine red cells. After 4 h of perfusion, red cells failed to support transport (adapted from Bucci *et al.*, 2003).

surrounding fluid before it reached the perfused tissue. Instead, cell-free carriers wetted the perfused membranes, bypassing red cells and the Ringer's fluid. The cell-free delivery was also helped by the facilitated oxygen diffusion (described by Wittemberg and Wittemberg, 2003) caused by the translational diffusion of hemoglobin molecules in solution. Red cells do not facilitate diffusion because their bulky size greatly retards their translational diffusion. These data were consistent with the observation reported above that, in the brain of exchange-transfused animals, cerebral blood flow was restored to normal during anemia by an amount of cell-free carrier much lower than the amount of missing red cell hemoglobin (Ulatowski *et al.*, 1996a).

ADIPYL-CROSSLINKED BOVINE HEMOGLOBIN

Searching for very low oxygen affinity molecules, by inserting a six-carbon-long adipyl crosslink into the β-cleft of bovine hemoglobin, we obtained two different compounds; one crosslinked between the two β1 Lysines (XL1), and another crosslinked between the β1 Valine and β81 Lysine of the opposite subunits (XL2) (Kwansa *et al.*, 2000). In isolated conditions, XL1 had a *P*50 near 50 mmHg with a Hill parameter near 1.5, while XL2 had a very high *P*50 (~100 mmHg; difficult to estimate) and a cooperativity of near 1.2. In these preparations, the amount of residual non-crosslinked hemoglobin was very small, and purification of dissociable material by affinity chromatography was not necessary. The fluid used for animal trials contained the two compounds in a 60 : 40 ratio in favor of XL1. The overall *P*50 of the fluid was near 60 mmHg. Only a few experiments were performed using these fluids because the exchange-transfused animals did not tolerate the infusion, possibly because of excess precapillary unloading of oxygen leading to enhanced vasoconstriction (unpublished). There was also little or no indication of oxygen delivery to tissues by the cell-free hemoglobins.

ZERO-LINK POLYMERIC HEMOGLOBIN

At this stage of development, we had learned that cell-free carriers are more efficient than red cells in delivering oxygen to tissues, that the relatively

high affinity of DBBF-BvHb does not appear to prevent release of oxygen to tissues, and that stabilized tetramers still extravasate and scavenge NO from the smooth muscle of the arterioles and produce hypertension. Thus, it was critical to avoid extravasation by increasing the size of the carriers to avoid filtration through the large pores of the capillary beds (Rippe and Haraldsson, 1994).

We obtained a large polymeric hemoglobin by activating the carboxyl groups on the surface of tetrameric hemoglobin, which in turn formed pseudopeptide bonds with the amino groups on the surface of nearby molecules. This polymeric hemoglobin was designated zero-link bovine hemoglobin (ZL-HbBv) because no crosslinking reagent remained between the tetramers. Residual low size polymeric species were eliminated by diafiltration with a 300-kDa NMW cut-off membrane. The resulting polymer had an average molecular weight near 25 000 kDa, with an estimated diameter of 300 nm. Its characteristics indicated an absence of oxygen-binding cooperativity and a *P*50 near 4 mmHg (Matheson *et al.*, 2002). ZL-HbBv did not extravasate and did not appear in the hilar lymph of the kidneys. As expected, it did not produce MAP increases when infused in mice, rats and cats (Figure 42.6). The half-time of intravascular retention was 10–12 h in the cat and 5 h in the mouse. In addition, transfusion of ZL-HbBv in unanesthetized cats caused no immediate change in MAP and no further changes over a 48-h observation period (Figure 42.6).

In other laboratories, large-volume hemoglobins have been obtained that do not produce MAP increases by substituting the surface Lysyl and cysteinyl residues with polyoxyethylene glycol (PEG). These hemoglobin molecules acquire a vast virtual volume, which may prevent extravasation. The reported oxygen-binding properties show a *P*50 near 6 mmHg and nearly null binding cooperativity (n = 1) (Vandegriff *et al.*, 2003).

Also, as discussed elsewhere in this book (see Chapter 32), large recombinant polymers (molecular weight near 1000 kDa) have been obtained by inserting cysteinyl residues on the surface of hemoglobin molecules, so as to form cystenyl bonds with adjacent molecules. They also combine a large size with oxygen affinity higher than that of red cells and exhibit loss of cooperativity (Fronticelli *et al.*, 2004).

It appears that most of these non-extravasating polymers have high oxygen affinity (i.e., higher than blood) and low cooperativity. Therefore, according to conventional view, they would not be physiologically competent. To the contrary, ZL-HbBv and the recombinant polymers were shown to deliver oxygen *in vivo* (Bucci *et al.*, 2001; Fronticelli *et al.*, 2004). The best evidence was the reduction of infarct size in mouse brains after a middle cerebral artery occlusion. In mice exchange-transfused with a 6-g/dl solution of ZL-HbBv, the infarct size was reduced by 40 per cent (Bucci *et al.*, 2001; Figure 42.7).

After 40 per cent exchange-transfusion of blood volume with ZL-HbBv in cats, cerebral blood flow was still well regulated at normal levels upon changes of perfusion pressure. Also, pial arterioles on the brain surface still dilated when hypercapnia was induced or acetylcholine was applied (Rebel *et al.*, 2003). However, the exchange transfusion itself resulted in a 20 per cent decrease in the diameter of brain pial arterioles without a

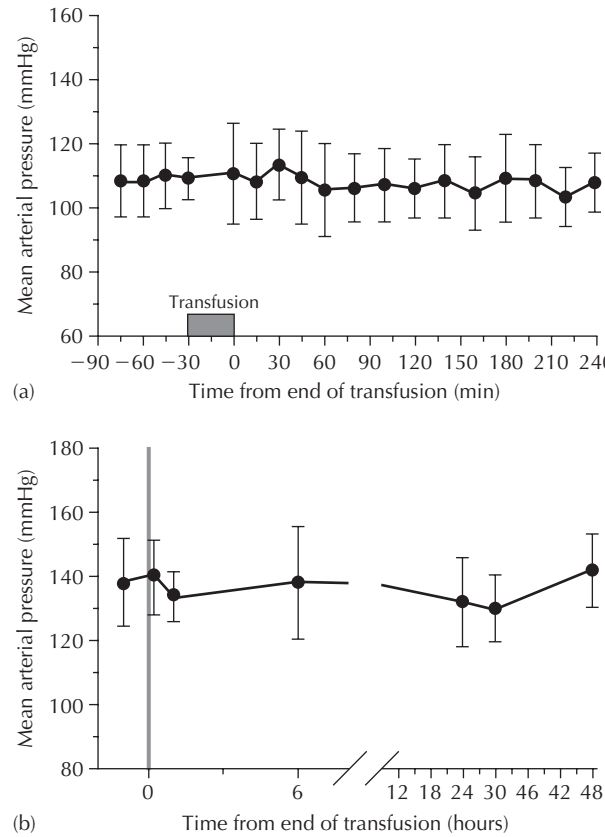

Figure 42.6 Exchange transfusion of ZL-HbBv produced no change in mean arterial pressure (± SD) over a 4-h observation period in anesthetized cats (a) or over a 48-h observation period in unanesthetized cats (b) (adapted from Matheson *et al.*, 2002).

change in cerebral blood flow. In the absence of increased MAP, this constriction was interpreted as a regulatory response to counteract the decreased blood viscosity, produced by a lower hematocrit. Thus, vasoconstriction prevented an increase in cerebral blood flow and the consequent hyperoxygenation of tissues. Conversely, when plasma viscosity was increased by addition of polyvinylpyrrolidone, the brain arterioles of animals transfused with ZL-HbBv dilated instead of constricting. Moreover, this vasodilation was much larger than in animals transfused with 5 g/dl albumin (Rebel *et al.*, 2003) (Figure 42.8). Therefore, pial arterioles constrict when blood viscosity is decreased and oxygen-carrying capacity is sustained by plasma-based polymeric hemoglobin, but these arterioles are capable of dilating during ZL-HbBv exchange transfusion when plasma viscosity is increased. These results indicate that brain arterioles autoregulate to changes in viscosity to keep oxygen transport well matched to oxygen demand. Furthermore, these results imply that ZL-HbBv delivers oxygen *in vivo* despite its low *P*50, even in anemic, non-severely ischemic tissues.

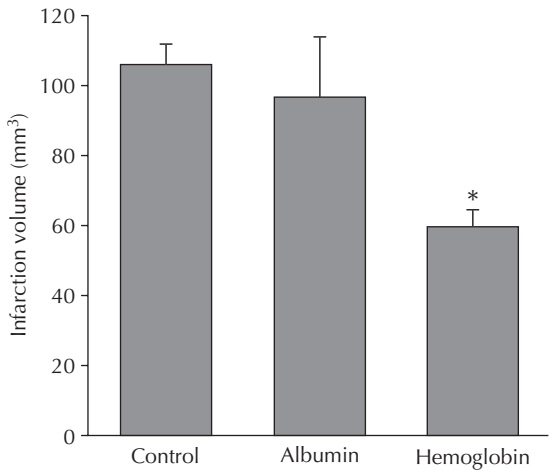

Figure 42.7 A 30 per cent exchange-transfusion of 6% ZL-HbBv polymer in mice during 2 h of middle cerebral artery occlusion reduced cerebral infarct volume compared to control and albumin-transfused mice.

One potential oxygen sensor is cytochrome P450 4A, localized in vascular smooth muscle. This P450 enzyme metabolizes arachidonic acid to 20-HETE, a potent vasoconstrictor, in an oxygen-dependent fashion over the physiological range of PO_2 (Harder *et al.*, 1996). When inhibitors of this pathway were applied to the pial surface, the constrictor response to ZL-HbBv exchange-transfusion was blocked (Koehler *et al.*, 2004). In contrast, NO synthase inhibition, which by itself produced constriction, did not completely block further constriction to ZL-HbBv transfusion. Thus, the constrictor response to decreased blood

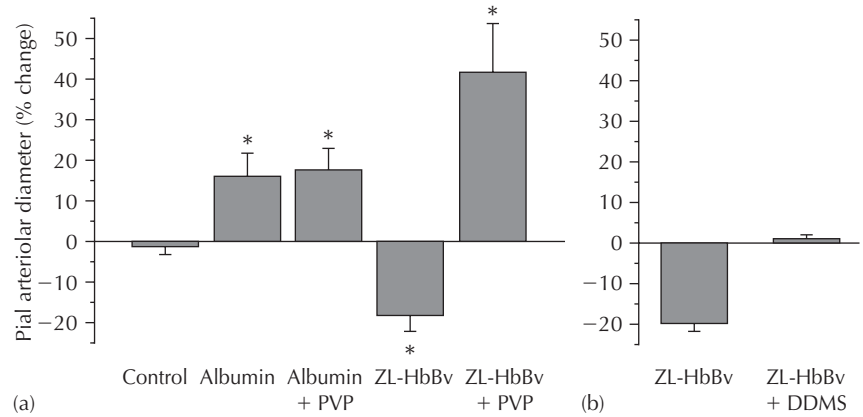

Figure 42.8 (a) Percentage change (± SE) in pial arteriolar diameter measured through a closed cranial window in anesthetized cats after a 40 per cent exchange-transfusion with 5% albumin or 6% ZL-HbBv. The constriction observed with ZL-HbBv at reduced hematocrit and blood viscosity was reversed to dilation in a group in which plasma viscosity was simultaneously increased by co-infusion of 20% polyvinylpyrrolidone (PVP). *$P < 0.05$ from time control group. (Adapted from Rebel *et al.*, 2003.) (b) The constrictor response to ZL-HbBv exchange-transfusion in anesthetized rats was blocked by superfusing the cranial window with the cytochrome P450 w-hydroxylase inhibitor *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS; 50 μM).

viscosity with sustained oxygen-carrying capacity is mediated primarily by the oxygen-sensitive P450 metabolism of arachidonic acid rather than NO scavenging.

OXYGEN DELIVERY BY HIGH AND LOW AFFINITY CARRIERS

The apparent paradox of oxygen delivery by high affinity carriers can be explained by simulating the oxygen-affinity dependence of the fractional oxygen delivery of the carriers. The simulations were performed using the standard Hill equation:

$$Y = PO_2^n / (P50^n + PO_2^n) \qquad 42.1$$

where Y is the fractional saturation of the carrier, PO_2 is the partial pressure of oxygen, and n is the cooperativity index. The fractional delivery was computed as the difference between Y at 100 mmHg (lungs) and Y at 2 mmHg (interstitial fluid; Wittemberg and Wittemberg, 2003). The results are shown in Figure 42.9. It is evident that, between 3 and 50 mmHg, the fractional delivery of the various carriers is very similar. Instead, there is a distinct dependence on binding cooperativity; the delivery is higher for n = 3 than for n = 1. Still, there is a substantial delivery even in the absence of cooperativity.

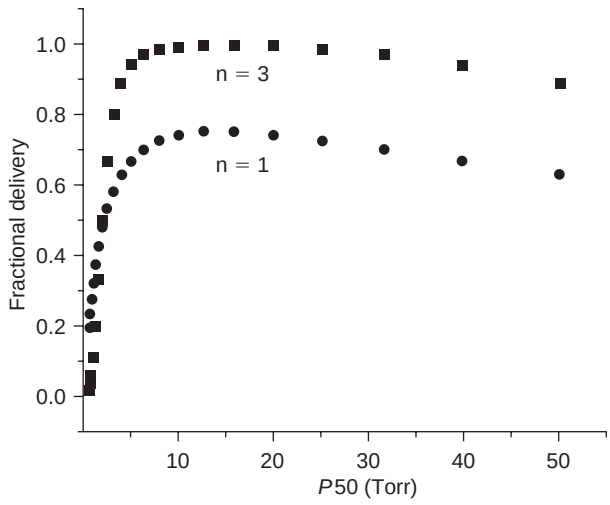

Figure 42.9 Dependence of the fractional oxygen delivery of carriers exposed to an oxygen gradient from 100 mmHg (at the lungs) to 2 mmHg (at the interstitial fluid).

SUMMARY

The availability of a variety of cell-free oxygen carriers (DBBF-BvHb, DECA, ZL-HbBv) and of diverse animal models (kidney function and lymph content, cranial windows, microsphere technology) produced a wealth of information which may help to solve, at least partially, the problems posed in the development of cell-free hemoglobin-based oxygen carriers.

Regarding the physiologic competence of oxygen affinity of the carriers, we have shown that all of our compounds participated in the microvascular response following their infusion (Ulatowski *et al.*, 1996a, 1996b, 1998; Asano *et al.*, 1998; Bucci *et al.*, 2001; Matheson *et al.*, 2002; Rebel *et al.*, 2003; Koehler *et al.*, 2004). Notably, their oxygen affinity spanned a *P*50 range between 4 mmHg (ZL-HbBv) and 30 mmHg (DECA), confirming the anticipation of the simulations in Figure 42.9.

It is interesting to note that oxygen delivery from cell-free carriers was more efficient than that from red cells (Bucci *et al.*, 2003), exposing tissues to the risk of hyperoxygenation. This stimulated a regulatory vasoconstriction, which did not produce MAP increase and was independent of NO production (Koehler *et al.*, 2004). All indications were that the oxygen delivered by the carrier (ZL-HbBv) participated in adjusting the rheology changes produced by either decreased or increased viscosity of blood, so as to control a normal oxygenation of tissues (Rebel *et al.*, 2003).

Notably, the regulatory vasoconstriction produced by infusion of ZL-HbBv was blocked when the production of 20-HETE by cytochrome P450 was inhibited (Koehler *et al.*, 2004). This phenomenon supports the hypothesis that cytochrome P450 is an oxygen sensor that plays an important role in blood flow regulation. This is novel information made possible only by the availability of a non-extravasating oxygen carrier, supporting the potential use of these carriers as means for investigating the physiology of oxygen transport *in vivo*.

Our data indicate that regulatory NO-independent vasoconstriction produced by hyperoxygenation does not produce MAP increase (Rebel *et al.*, 2003) and results in normoxia, without depriving tissues of oxygen, as previously considered (Vandegriff and Winslow, 1995). We also presented strong evidence that luminal scavenging of NO does not produce

profound systemic vasoconstriction, suggesting that any intraluminal scavenging of NO is inadequate to reduce NO on the abluminal side, as proposed in the simulations of Kavdia and Popel (2004). This discrepancy may be resolved, assuming that there is limited diffusion of NO through the arterial walls that separate their luminal and abluminal sides.

That MAP increase is inversely proportional to the molecular size and volume of the infused carrier is well documented (Sakai *et al.*, 2000). Analysis of the lymph content of hemoglobin demonstrated that extravasation is a critical parameter in designing cell-free oxygen carriers. In this regard, the most used technique is to transform tetrameric hemoglobin into large polymeric molecules. Inevitably, the chemical treatments used for polymerizing hemoglobin produce a distribution of molecular sizes, leaving behind some low- and non-polymerized species. We successfully used diafiltration with a 300-NMW cut-off membrane to eliminate these low molecular weight species from the transfusing fluids. Knowledge of the molecular distribution of ZL-HbBv in solution, and its failure to appear in the lymph, allowed a much better and clearer interpretation of the microvascular activity following its infusion.

Our data seem to suggest that, as long as the carrier does not extravasate, all other characteristics are flexible. Actually, this potential diversification may result in a broader medical and clinical applicability than just crude blood or red cell replacement. The absence of extravasation and the increased MAP allow for assessment of the *in vivo* competence of the carriers independent of the death/survival statistics of infused animals or humans (Saxena *et al.*, 1999; Sloan *et al.*, 1999). Possibly, their use for investigating oxygen transport *in vivo* will result in the development of novel clinical and therapeutic procedures. These are the most challenging and interesting aspects of future evolutions of this technology.

EDITOR'S SUMMARY

A systematic approach to the problem of optimal oxygen affinity and molecular size was undertaken to include study with 'tetrameric' and polymerized hemoglobins with different properties and sizes. The different molecules were characterized and then tested in a variety of animal models.

This research group concluded that prevention of extravasation is of paramount importance; they postulate that in the interstitial space hemoglobin can very efficiently scavenge NO, a natural vasorelaxant, resulting in hypertension. They also conclude that cell-free hemoglobin, regardless of *P*50, is highly efficient at delivery of oxygen to vessel walls because of its participation in facilitated diffusion, and can produce vasoconstriction independently of NO scavenging. However, they postulate that this is a separate mechanism that does not produce hypertension.

A very interesting product of this research is 'zero-linked' hemoglobin, so called because it results from direct polymerization of surface amino acids rather than the use of crosslinking agents. This product has a very large molecular volume and high oxygen affinity. It is a very useful model compound to explore important physical properties and their effect on biological reactivity.

ACKNOWLEDGMENTS

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SECTION

7

Liposomes and Related Products

A variety of products, not based primarily on either the perfluorocarbon or the hemoglobin approach, have also been developed, but these remain in the early stages of preclinical development either because the technology is more difficult or because the cost is too high for mass production. Nevertheless, experience with these products is instructive. In particular, considerable effort has been expended to produce truly artificial red blood cells, based on liposome spheres that contain hemoglobin in their central cavity. Such a product has the potential for longer intravascular retention as well as protection of tissue from the effects of oxidative reactions.

Chapter 43

Liposome-Encapsulated Hemoglobin: History, Preparation and Evaluation

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HISTORICAL PERSPECTIVE

The development of a readily available oxygen-carrying resuscitative fluid for administration to victims of acute hemorrhagic shock has been a long-sought goal. An ideal oxygen-carrying resuscitative fluid should emulate as many of the functions of fresh whole blood as possible. It should have the capacity to carry and deliver oxygen, like red blood cells (RBCs), as well as provide oncotic activity to maintain circulatory filling pressure. Like RBCs, it should remain in the intravascular space for a prolonged time period without any toxicity or immunogenicity. This oxygen-carrying resuscitative fluid may also contain long-circulating therapeutic agents to address other pathophysiologic alterations that occur during hemorrhagic shock and resuscitation, such as complement activation and reperfusion injury. In addition, the product should be shelf-stable, easy to use and moderate in cost.

The concept of hemoglobin encapsulation within an artificial membrane was first investigated by Chang in 1964 (Chang, 1964). Since then, nanocapsular and liposome-encapsulated hemoglobin (LEH) products have been developed and currently are in the preclinical phase. Compared to free hemoglobin products, encapsulated hemoglobin has several important advantages, including:

1. A longer circulation time
2. Reduced vasoactivity
3. The potential to co-encapsulate allosteric modifiers and antioxidants
4. Oxygen diffusivity closer to that of RBCs
5. Metabolism by the reticuloendothelial system (RES) of the liver and spleen in a similar fashion to RBCs.

Since vertebrate hemoglobin has evolved into a spatially isolated existence inside the RBCs, a more complete oxygen carrier would be hemoglobin encapsulated within a synthetic envelope that also contains the oxido-reductive system of naturally occurring RBCs. Free hemoglobin acts as a pro-inflammatory protein that may be detrimental if administered for resuscitation in traumatic brain injury (Gibson *et al.*, 2002). Autoxidation of hemoglobin produces harmful reactive oxygen species (Kawano and Hosoya, 2002), and therefore, hemoglobin has been called an oxidative neurotoxin. It may contribute to brain cell injury after brain trauma and hemorrhagic shock (Regan and Rogers, 2003). Products that encapsulate hemoglobin are less likely to have these pro-inflammatory effects. Encapsulated hemoglobin

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has also been shown to be devoid of the vasoconstrictive effects that are commonly seen with molecularly modified free hemoglobin (Nakai *et al.*, 1994; Rudolph *et al.*, 1997).

Historically, encapsulation of hemoglobin inside liposomes has a distinguished record of development (Djordjevich and Miller, 1980; Gaber *et al.*, 1983; Farmer *et al.*, 1988; Rudolph, 1988; Rabinovici *et al.*, 1992; Rudolph, 1994; Tsuchida, 1998). In the USA, the major impetus for LEH research came from a broad approach of the USA Army and Navy over the past 20 years to develop artificial blood substitutes for battlefield resuscitation. Early in the development process, hemoglobin encapsulation into liposomes was met with the same challenges common to all liposome research (Bangham, 1992). Researchers noted that liposomes were recognized by the RES and removed from circulation to different degrees depending on the size and the lipid composition. Another challenge was the need to develop a large-scale production technology without altering the functionality of the encapsulated hemoglobin. These obstacles were overcome by the development of a high-shear method to encapsulate hemoglobin inside lipid bilayers (Beissinger *et al.*, 1986, Farmer and Gaber, 1987) and the insertion of polyethylene glycol-lipid in the bilayer (Phillips *et al.*, 1999). Since these early days the methods of hemoglobin encapsulation have improved so that it is now possible to encapsulate large amounts of hemoglobin in a stable and functional form. Products that encapsulate hemoglobin within phospholipid membranes are variably called hemoglobin vesicles (HbV), neo-red cells (NRC) or LEH (Figure 43.1). These liposome formulations encapsulate highly concentrated and purified hemoglobin (>36 g/dl).

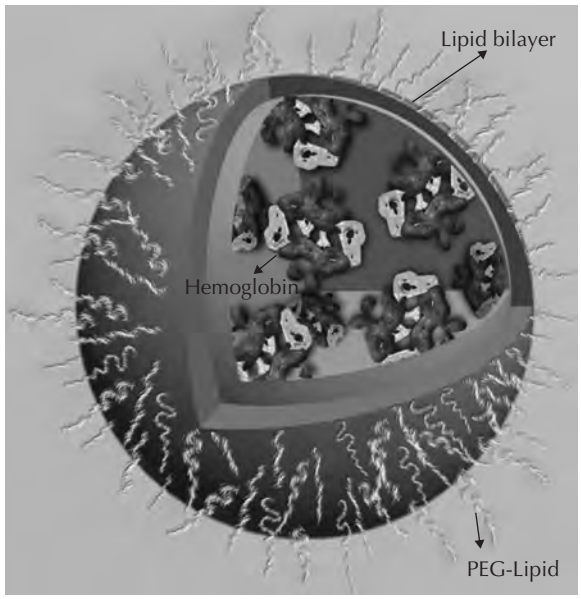

Figure 43.1 An illustration of liposome-encapsulated hemoglobin. See color plate 20.

PREPARATION OF LIPOSOME-ENCAPSULATED HEMOGLOBIN

Lipid composition and charge

The lipid composition of LEH is based on a combination of a saturated high-carbon phospholipid and cholesterol. The content of cholesterol in LEH is maintained at around 40–50 per cent of a molar phospholipid concentration. The choice of a saturated high-carbon phospholipid has been mostly between distearoylphosphatidylcholine (DSPC) and dipalmitoylphosphatidylcholine (DPPC). These two lipids differ in their phase transition temperature, which dictates the manufacturing method required for LEH and its *in vivo* behavior. DSPC melts at 55°C and therefore, compared to DPPC (which melts at 41°C), it is more stable *in vivo*. At the same time, DPPC LEH can be processed by methods that require less shear force to reduce particle size. Thus, DPPC containing LEH may be produced by extrusion of a hemoglobin–lipid mixture at about 41°C through filters of well-defined pore sizes (Sou *et al.*, 2003). On the other hand, DSPC LEH requires greater shear force to reduce its particle size to optimum level, and therefore microfluidization is the preferred process.

A major problem in LEH manufacturing is the low encapsulation efficiency of hemoglobin inside the liposomes. It is desirable to encapsulate large amounts of hemoglobin within a minimum amount of lipid (hemoglobin-to-lipid ratio, Hb:L). To increase the encapsulation of proteins inside liposomes, anionic lipids such as dimyristoyl- and dipalmitoyl-phosphatidyl glycerol (DMPG and DPPG) are usually incorporated in the formulation (Drummond *et al.*, 1999; Walde and Ichikawa, 2001). Tsuchida and coworkers achieved a hemoglobin-to-lipid ratio of 1.61 using DPPG/DPPC/cholesterol (1:5:5, molar ratio) in conjunction with optimal encapsulating conditions (Sakai *et al.*, 1996; Takeoka *et al.*, 1996). Charged lipids enhance encapsulation efficiency by interacting with oppositely charged domains of

proteins. However, anionic liposomes are known to interact undesirably *in vivo* with complement and other circulating proteins (Cullis *et al.*, 1998; Miller *et al.*, 1998; Szebeni, 1998). Such interactions result in a rapid uptake of LEH by the RES, and toxic effects, such as pseudoallergy which is manifested as vasoconstriction, pulmonary hypertension, dyspnea, etc. A fine balance has to be accomplished between the necessity of maximizing hemoglobin encapsulation in the least amount of lipid using anionic lipids, and the importance of keeping charge-associated undesirable effects in check. In this regard, it is important to mention a recently developed synthetic anionic lipid, 1,5-dipalmitoyl-l-glutamate-N-succinic acid, and its use in LEH (Sou *et al.*, 2003). The investigators believe that this particular lipid is better tolerated than anionic phospholipids, though a concern about its metabolism after administration still remains unresolved. Recently, our group has reported a comparative study on neutral and anionic LEH (Awasthi *et al.*, 2004a). As shown in Figure 43.2, inclusion of anionic lipid in the LEH composition had a significant effect on LEH retention in blood, yet the encapsulation efficiency of hemoglobin was not significantly different among the preparations (Awasthi *et al.*, 2004a).

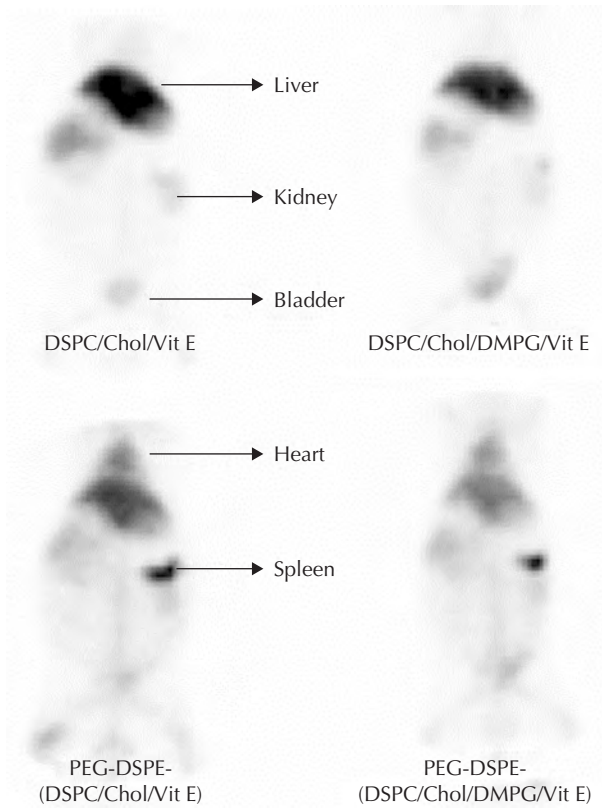

Figure 43.2 Gamma camera images acquired 24 h after rabbits were intravenously injected with technetium-99m-labeled LEH (15 mg phospholipid in 2 ml). Note the large amount of PEG-LEH in heart, reflecting circulating LEH, compared to non-PEGylated LEH. The anionic charge has significant impact on circulation kinetics of LEH.

Particle size

As an oxygen carrier, it is desirable that LEH has a long circulation time in blood without significant accumulation in any other organ. Liposome size and encapsulation efficiency are critical factors governing the utility of LEH. In general, the circulation half-life ($T_{1/2}$) of conventional liposomes decreases with increasing size, anionic charge density and bilayer fluidity. However, liposome size and surface charge override the state of bilayer stability in determining liposome clearance *in vivo* (Gregoriadis, 1995). Large liposomes (>200 nm) have short circulation $T_{1/2}$, as they are rapidly eliminated from circulation by the RES through a complement-mediated phenomenon (Bradley *et al.*, 1998; Szebeni, 1998; Szebeni *et al.*, 2000). A liposome of large size may facilitate multi-ligand interactions, or may be opsonized by a protein specific for large liposomes (Harashima *et al.*, 1994). On the other hand, small liposomes (<200 nm) circumvent RES uptake due to their reduced recognition by circulating opsonins. At the other extreme, very small liposomes (~60 nm) accumulate in liver more than the intermediate-sized liposomes (Abra and Hunt, 1982; Scherphof, 1991; Awasthi *et al.*, 2003). It appears that elevated liver accumulation of very small liposomes is due to their access to the hepatocytes through the fenestrated hepatic endothelium (Liu *et al.*, 1992). Incorporation of polyethylene glycol (PEG) lipids in the liposomes prevents liposome-induced complement activation (Ahl *et al.*, 1997; Bradley *et al.*, 1998) and reduces the dependence on size for prolonged circulation.

Liposome size is also an important determinant of encapsulation efficiency because as the size of the liposomes increases, the entrapped volume increases for constant lamellarity (Perkins *et al.*, 1993). PEG lipids reduce the requirement of small size for long circulation, but their influence is restricted within a size range. The ideal LEH formulation has a size that is as large as possible while still retaining a PEG-mediated prolonged

circulation $T_{1/2}$. Beyond a certain size, the stealth property of PEG-liposomes becomes insignificant and the distribution is characterized by proportionately high RES accumulation. For LEH, it seems that a size range of 210–275 nm is optimum, where PEG liposomes still retain prolonged circulation (Awasthi *et al.*, 2003). Above this size range the circulation $T_{1/2}$ is limited, while below this range the captured volume is considerably reduced. Earlier, Maruyama *et al.* (1992) showed that the circulation $T_{1/2}$ of DSPC vesicles drops off rapidly over 300 nm.

Hemoglobin source

As with any other hemoglobin-based oxygen carrier (HBOC), the choice of hemoglobin for LEH is limited to that of human or bovine origin. Currently, stroma-free human hemoglobin from outdated RBCs is the source of hemoglobin for most LEH research and development around the world. However, during the early stages of development, purified bovine hemoglobin was investigated for preparing LEH (Fronticelli *et al.*, 1986; Ligler *et al.*, 1989; Rudolph *et al.*, 1991; Mobed *et al.*, 1992). The deoxygenated and carbonylated bovine hemoglobin preparations have denaturation transition temperatures of 83°C and 87°C, respectively, which are higher than those of human hemoglobin and enable pasteurization (Sakai *et al.*, 2002). It is also relatively easy to regulate the oxygen affinity of bovine hemoglobin because of its responsiveness to chloride ion. With all these advantages, bovine hemoglobin appears to be a convenient and abundant choice for LEH preparation. However, immunological response to multiple infusions of bovine hemoglobin-based LEH is still a concern, although it has been shown that antibody response does not affect the oxygen-binding properties of bovine hemoglobin in dogs (Hamilton *et al.*, 2001). Converting from human to bovine sources of hemoglobin could also have significant advantages in terms of the economics of LEH production. At this point, it is fair to acknowledge recombinant hemoglobin as a potential source of hemoglobin for LEH. In an alternative innovative approach, Kobayashi *et al.* (1995) have attempted to transform the protoheme moiety of the hemoglobin molecule into an amphiphilic compound. The amphiphilicity imparted to the protoheme enables it to interact with phospholipid membranes of liposome for efficient encapsulation, besides protecting it from oxidative degradation.

PEGylation of LEH

One way to circumvent the physiological responses to LEH administration is to conceal the liposome surface with hydrophilic polymers by incorporating PEG-linked phosphatidylethanolamines (PEG-PE) in the bilayer structure. It is believed that a hydrophilic PEG coating on the liposome surface creates a steric barrier, enabling liposomes to circulate longer (Torchilin and Papisov, 1994). Incorporation of PEG-PE in the liposome bilayer is most easily done when preparing the lipid phase just prior to its hydration with an aqueous phase (Phillips *et al.*, 1999). However, this technique results in the PEG brush or mushroom occupying the limited space inside the liposomes. In addition, the same steric hindrance that makes PEG useful for prolonged circulation may inhibit the encapsulation of substances by exclusion phenomena (Nicholas *et al.*, 2000). The smaller the size of the liposomes, the greater is the impact of PEG on the total usable space for encapsulated material. This conventional method of PEGylation requires more PEG lipid than is needed for useful stealthing of a liposome. In the case of multi-lamellar liposomes, the magnitude of wastage is greater (Awasthi *et al.*, 2004a). Realization of the problems associated with conventional PEGylation led to a technique where PEG-PE is inserted in the outer layer of liposomes after following the final manufacturing stages (Uster *et al.*, 1996). This technique, called post-insertion, is especially useful in the case of LEH. Basically, amphiphilic PEG-PE exists as a monomer below its critical micelle concentration, and when mixed with preformed LEH it intercalates into the outer lipid layer of LEH. The degree of incorporation is a function of PEG-chain length, fatty acid, temperature and concentration of lipids (Sou *et al.*, 2000). Besides doubling the circulation $T_{1/2}$ of LEH and reducing accumulation in the RES, this post-insertion technique improves the encapsulation efficiency of hemoglobin (Awasthi *et al.*, 2004a).

PROPERTIES

Oxygen affinity

Oxygen affinity is measured as the partial pressure of oxygen to saturate 50 per cent of hemoglobin (*P*50). Normal *P*50 of human hemoglobin

in RBCs is about 27 mmHg (Bunn and Forget, 1986). Certain substances called allosteric modifiers, such as 2,3-diphosphoglycerate (2,3-DPG), affect the oxygen affinity of hemoglobin (Miller *et al.*, 1970). Other substances with hemoglobin-modifying properties similar to 2,3-DPG are pyridoxal 5′-phosphate (PLP) and inositol phosphate (IP) (Goodford *et al.*, 1978). A synthetic compound, RSR13, is also capable of changing hemoglobin oxygen affinity (Miyake *et al.*, 2003). Hemoglobin oxygen affinity in LEH is easily altered by co-encapsulation of PLP (Sakai *et al.*, 2000; Sou *et al.*, 2003) or IP (Usuba and Motoki, 1995). Although it has long been believed that artificially assembled HBOCs should have a *P*50 close to that of RBCs, recent findings have supported the use of low *P*50 HBOCs in severe blood loss. It has been demonstrated that under mild hypoxia a high *P*50 may be helpful, while in severe hypoxia a low *P*50 may be beneficial (Kavdia *et al.*, 2002). Animal experiments also support this view (Eichelbronner *et al.*, 2002; Baines and Ho, 2003; Shirasawa *et al.*, 2003; Winslow and Kramer, 2003). Interestingly, LEH with a low affinity has also been found to have improved oxygen delivery and functional capillary density (Sakai *et al.*, 1999).

Viscosity

The rheologic property of blood plays an important role in regulation of physiology and pathophysiology of the cardiovascular system. HBOCs in solution show Newtonian flow, but LEH, like whole blood, demonstrates non-Newtonian flow behavior (Sakai *et al.*, 2000). LEH is particulate in nature, and therefore has a lower viscosity compared to the equivalent concentration of free hemoglobin solution. In practice, the viscosity of LEH is imparted by suspending it in plasma expanders such as albumin or hetastarch. When the albumin concentration is adjusted to about 5 per cent (Chung and Wang, 1997), the viscosity of the LEH/albumin suspension is similar to that of human blood. It is recommended that the viscosity of an HBOC/blood mixture should be at least 2 cPs in order to ensure normal microvascular function (Intaglietta, 1999). At a certain level of hemodilution, enhancing viscosity improves the microcirculation by directly influencing the vascular diameter via endothelial wall shear stress (Muizelaar *et al.*, 1986). However, too viscous an LEH/plasma expander suspension may result in aggregation and capillary blockage, and may also increase the diffusion barrier for the flux of oxygen from LEH to the tissues.

Colloid oncotic pressure and isotonicity

Blood is a complex system with a variety of circulating proteins that not only enhance the viscosity of blood but also exert oncotic pressure in the vasculature. Oncotic pressure acts in opposition to the hydrostatic pressure, and balances the fluid distribution between blood and interstitial fluid compartments. Normal plasma has a colloid oncotic pressure (COP) of about 28 mmHg (Weil *et al.*, 1979; Roberts and Bratton, 1998). Unencapsulated hemoglobin solutions exert oncotic pressure by virtue of their high hemoglobin concentration, and any modification of hemoglobin has a significant impact on its oncotic property (Vandegriff *et al.*, 1997). LEH by itself has poor oncotic pressure, but it is made oncotically active by adding albumin, pentastarch or other such substance to the dispersion medium. An adequate oncotic activity in LEH is necessary to correct and maintain the vascular volume deficit.

Like COP, osmotic pressure is also a colligative property and depends on the number of species in solution. Although closely related to the COP, it is a different physical property. Osmotic properties of LEH depend upon the dispersion medium, which usually contains salts and oncotic agent. Hemoglobin, once encapsulated, has an insignificant impact on both the osmolarity and the oncotic activity of LEH. Intuitively, a resuscitative fluid containing LEH should be isotonic with plasma since it will be administered in large quantities. However, in the context of recent evidence gathered for hypertonic saline as a resuscitative fluid, this may not be always the case (Mazzoni *et al.*, 1988; Kramer, 2003; Pascual *et al.*, 2003). The widespread salutary effects of hypertonic saline have made this fluid an attractive choice, especially when combined with hyperoncotic colloid (Kramer, 2003; Moore *et al.*, 2004). Indeed, a combination of LEH and hypertonic saline was found to be associated with improved blood pressure, reduced acidosis and increased survival in a rat model of 70 per cent hypovolemic shock (Rabinovici *et al.*, 1993).

ENDOTOXIN INTERACTION

Just as with any HBOC, contamination with endotoxin is a significant and frustrating problem in

LEH manufacturing. It is more critical in LEH for two reasons. First, hemoglobin has a strong tendency to bind endotoxin, where one hemoglobin molecule binds to four endotoxin molecules (K_d 3.1×10^{-8}M; Jurgens *et al.*, 2001). Second, endotoxin has amphiphilicity that enables its stable insertion into the lipid bilayer. The best possible way to prevent endotoxin contamination is to use aseptic precautions with utmost care. All the machinery, filters and water should be endotoxin-free. Glass and metallic components may be dry-heat sterilized at about 200°C for 3–4 hours, while components that cannot be subjected to dry heat must be autoclaved, washed with 0.2–0.5 M sodium hydroxide solution and flushed with endotoxin-free water. For endotoxin testing, the common *Limulus* amebocyte lysate (LAL)-based methods are not applicable in HBOCs, unless the assay is modified to account for the presence of hemoglobin (Vanhaecke *et al.*, 1987; Roth and Levin, 1994; Cliff *et al.*, 1995). By compensating for hemoglobin absorbance and by diluting the sample to maximum valid dilution (MVD), it is sometimes possible to use chromogenic methods for endotoxin testing in samples with small amounts of hemoglobin. For highly concentrated preparations of hemoglobin, however, dilution to MVD may not work, and a kinetic gel clot method based on LAL may be the method of choice. The gel clot method is relatively insensitive to the presence of hemoglobin at MVD, and can be used effectively to monitor endotoxin (Cliff *et al.*, 1995). Further modifications of the gel clot assay for endotoxin determination in LEH have been proposed, which use a proper detergent to solubilize the lipid bilayer (Harmon *et al.*, 1997) and a histidine-immobilized agarose gel to concentrate LPS (Sakai *et al.*, 2004a).

CIRCULATION TIME

Following acute blood loss, the body takes 5–7 days to replace lost volume and oxygen-carrying capacity (Hughes *et al.*, 1995). The desired intravascular persistence of an oxygen carrier should be at least equal to the time required to regenerate RBCs (Sehgal *et al.*, 1984). Transfused RBCs remain functional in circulation for many days, but the same cannot be said for HBOCs, including LEH. It is a significant challenge to formulate an LEH that matches the functional efficacy of RBCs (Phillips *et al.*, 1997a). Two continuous phenomena govern *in vivo* efficacy of LEH: elimination from circulation via usual RES-mediated metabolic pathways, and rapid conversion of hemoglobin to methemoglobin. Once administered, soluble hemoglobin converts to methemoglobin at a rate of $>$1–2 per cent per hour (Ogata *et al.*, 1997). Hemoglobin within the RBCs maintains its functionality by the support of all the protective mechanisms provided inside the RBC. LEH can partially imitate RBCs by employing co-encapsulated enzymes and co-factors. In reality, however, even co-encapsulation of catalase or other enzymes may not impart permanent stability and only delays the eventual oxidation of hemoglobin to non-functional methemoglobin (Teramura *et al.*, 2003). Soluble HBOCs are also removed from circulation by their tendency to extravasate in the interstitial space. The resulting loss of circulating HBOC contributes to its short circulation $T_{1/2}$. Encapsulation of hemoglobin eliminates the problem of dissociation and extravasation.

Although free hemoglobin in circulation has its own metabolic pathway, once encapsulated its fate is governed by the disposition of liposomes. Using nuclear imaging and biodistribution techniques, it has been possible to visualize that the liver and spleen are the major organs of LEH accumulation (Goins *et al.*, 1995; Phillips *et al.*, 1999; Goins and Phillips, 2001). These two organs are responsible for the metabolism of normal liposomes, and their relative contribution to LEH metabolism is a function of liposome size, composition, and the presence of surface PEG coating. PEG-LEH shows significantly less RES accumulation and longer circulation $T_{1/2}$ in animals than does LEH without PEG lipid (See Figure 43.2). When infused in rodent models of 25 per cent hypovolemic shock, circulation $T_{1/2}$ of LEH was 30 and 39 hours in rats and rabbits, respectively (Awasthi *et al.*, 2004b).

Another factor that influences the circulation $T_{1/2}$ of LEH is the amount administered. It has been shown that lipid dose determines the extent of circulation of liposomes (Laverman *et al.*, 2000). A large dose of liposomes saturates the endocytotic pathways or the plasma opsonizing factors and tends to increase the circulating liposomes in blood (Drummond *et al.*, 1999). Such would be the case of LEH where several grams of lipid would be infused as part of a resuscitative fluid to correct oxygen deficit. Although it is difficult to accurately reflect the circulation $T_{1/2}$ of LEH in humans on the basis of animal experiments, it has been roughly

estimated that a circulation $T_{1/2}$ of 12–20 hours in rats or mice translates into 40–60 hours in humans (Woodle *et al.*, 1995). Based on this relationship, the 30-h $T_{1/2}$ of the current formulation of LEH in rats would likely translate to a $T_{1/2}$ of over 90 hours in humans.

TOXICITY

Acellular hemoglobins have several untoward effects related to their tendency to extravasate through the endothelium and sequester nitric oxide (NO), resulting in vasoconstriction (Creteur *et al.*, 2000; Riess, 2001; Lane and Gross, 2002; Winslow, 2003). LEH has no tendency to extravasate, but it has its own toxicity profile. It has been found that LEH induces toxic reactions mediated by complements. Historically, liposomal formulations have been found to cause pulmonary hypertension and other hypersensitivity reactions in patients (Parnham and Wetzig, 1993; Szebeni *et al.*, 2000; Chanan-Khan *et al.*, 2003). LEH-mediated complement activation is dependent on the particle size, size distribution, charge and surface coating. A marked reduction in circulating thrombocytes has also been observed in animals (Phillips *et al.*, 1997a). LEH containing anionic lipid causes significantly more severe thrombocytopenia than that induced by neutral LEH (Figure 43.3). These effects have been linked to complement activation, and are grouped within complement activation-related pseudoallergy (Szebeni, 1998; Szebeni *et al.*, 2000). Complement activation may be partially resolved by PEGylation, control of size distribution, and reducing the content of anionic lipids. However, recently it has been found that hemorrhage alone may trigger complement activation regardless of the type of resuscitation (Szebeni *et al.*, 2003). Acidosis, endotoxemia and other ischemia-related tissue alterations may be the associated triggers (Szebeni *et al.*, 2003).

Another obvious concern of RES toxicity while handling large amounts of particulate lipid was allayed when it was observed that LEH caused only a transient change in the plasma enzyme profile in rats without any irreversible damage to the RES (Sakai *et al.*, 2001, 2004b).

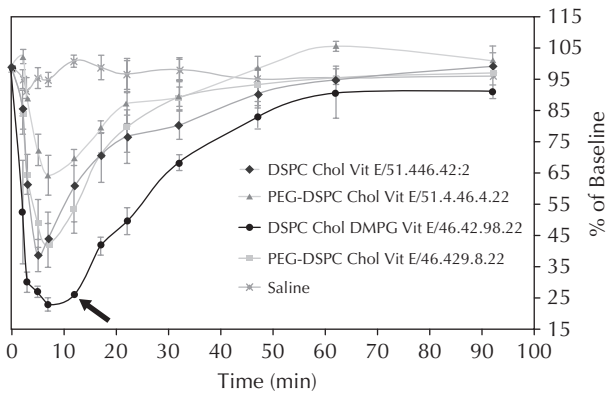

Figure 43.3 LEH induces transient but rapid thrombocytopenia in rabbits, which is partially mitigated by PEGylation. The extent of platelet-drop (arrow) is a function of charge on the LEH surface. Rabbits were injected with indium-111-labeled platelets, and a small dose of LEH (~4 mg phospholipid, 0.5 ml) was administered after 30 min. Circulation of In-111-labeled platelets was followed by withdrawing arterial blood at various times and counting the radioactivity in a well counter.

OXYGEN DELIVERY

Several physiological parameters are generally monitored when oxygen carriers are evaluated in animals for providing information about the shock status, but all these parameters are indirect measures of oxygen delivery and metabolism. Monitoring blood flow and hemodynamic status alone may not be sufficient in hemorrhagic shock. Oxygen delivery may be compromised even in the presence of normal cardiac output, blood pressure and cardiac filling pressure, due to compensatory mechanisms in response to shock. Determination of oxygen debt that may be accumulating is critical for appropriate assessment and evaluation of the effectiveness of resuscitation, so that onset of multiple organ failure is prevented. Therefore, real-time oxygen delivery, oxygen consumption and tissue acidosis in the critical organs, such as brain, are the important processes that should be monitored while evaluating resuscitation.

Our group has been using nuclear medicine techniques to investigate the oxygen-carrying capacity of LEH (Phillips *et al.*, 1997b; Goins *et al.*, 1998). Recent development of a small animal positron emission tomography (PET) device has enabled visualization of changes in cerebral blood flow, cerebral blood volume and cerebral metabolic rate of oxygen. The technique is based on the administration of oxygen-15 (O-15) labeled water, carbon monoxide (CO) and oxygen (O_2) in

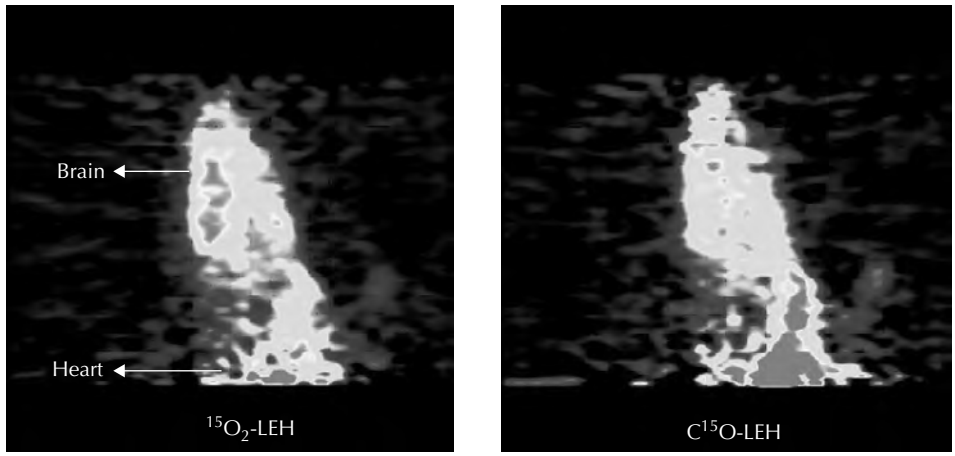

Figure 43.4 LEH was loaded with O_2 gas or CO (both labeled with O-15) and intravenously injected in a normal rat. Sagittal sections of PET images of rat brain are shown. The image acquisition was initiated with administration of labeled LEH and continued for 7 minutes. LEH releases O_2 to the brain tissue as indicated by the intensity of the brain image, but CO-labeled LEH predominantly shows blood volume in the brain. The degree of oxygen accumulation increases in the following order of color intensity: purple < blue < green < yellow < red. See color plate 21.

tracer quantities. With the short physical $T_{1/2}$ (2 minutes) of O-15, it is possible to perform serial tomographic imaging of the same animal at various stages – e.g. baseline, after hemorrhagic shock, and post-resuscitation. Using the same animal for all three stages of an experiment enables negation of variation between different animals. O-15 acts as a physiological tracer for oxygen, and its binding kinetics to hemoglobin is identical to that of non-radioactive O-16. Figure 43.4 shows an example of a PET image demonstrating cerebral oxygen delivery by LEH. This technique is proving to be very powerful, and it has the potential to evaluate the effectiveness not only of oxygen carriers but also of resuscitative fluids and protocols in general. Furthermore, it appears to be a useful tool for providing new basic insights into the pathophysiology of hemorrhagic shock.

STORAGE STABILITY

The storage shelf-life of an LEH preparation is a function of two factors: maintenance of the size of the LEH, and prevention of oxidation of the lipid and hemoglobin. Both of these aspects are influenced by the storage conditions – the temperature and the inertness of the atmosphere. LEH suspension tends to settle on storage unless the particle–particle interaction is inhibited by a steric factor or charge-based repulsion. Inclusion of a suitable PEG lipid provides steric inhibition, while incorporation of anionic lipid improves storage stability by repulsion among LEH particles in suspension. Experience suggests that less than 2 mole% of anionic lipid and about 0.3 per cent post-inserted PEG lipid (Sakai *et al.*, 2000) is capable of inhibiting LEH sedimentation. As far as oxidative degradation of LEH is concerned, both hemoglobin and phospholipids are sensitive to oxidation, and their oxidative products are mutually destructive (Takeoka *et al.*, 2002). The question of lipid oxidation has been addressed in detail several times, and it has been shown that inclusion of lipophilic vitamin E retards lipid peroxidation by free radicals (McCay, 1985; Chatterjee and Agarwal, 1988; Grit and Crommelin, 1993). The oxidation reaction is temperature-sensitive, and therefore it has been recommended that the LEH should be stored at 4°C. Hemoglobin, also, oxidizes to methemoglobin in a temperature-sensitive reaction; about 50 per cent of stroma-free hemoglobin turns into methemoglobin when incubated in ambient air at 37°C. The reaction is slowed if the LEH is deoxygenated by purging it with an inert gas (nitrogen) during storage. Such preparations have been shown to be amenable to storage even at room temperature (25°C) for a prolonged duration. A long-sought goal is the ability to freeze-dry LEH for ease of transportation and enhanced stability

(Rudolph, 1988, 1994), but a detailed discussion of this aspect is beyond the scope of this chapter. It is sufficient to mention that hemoglobin stability (Labrude *et al.*, 1984; Heller *et al.*, 1999), as well as liposome size and stability (Rudolph and Cliff, 1990; Crowe and Crowe, 1992) are problem areas in the LEH freeze-drying process.

SUMMARY

LEH appears to have many of the advantageous properties of RBCs, yet, like other HBOCs, it is not a complete resuscitation fluid for shock. Intervention in severe hemorrhage requires more than the provision of enhanced oxygen-carrying capacity. Functional imaging using PET suggests that the oxygen carriers should also be able to deliver oxygen for its consumption in cellular metabolism. At the same time, pathophysiology related to hemorrhage calls for additional components in LEH formulation. Experience to date suggests that a complex LEH formulation containing inhibitors of complement activation and reperfusion injury, oncotic substance, long circulation and functional $T_{1/2}$, while being stable at wide range of temperatures and having minimal toxicity, needs further development. Still, many of the important milestones have been accomplished and LEH continues to have great potential as part of an advanced oxygen-carrying resuscitative fluid.

EDITOR'S SUMMARY

In many ways, liposome-encapsulated hemoglobin (LEH) can be considered the nearest to 'artificial red cells' of any of the products currently under development. It consists of an artificial sphere, made up of phospholipids, which contains hemoglobin in some form in the center. Compared to cell-free hemoglobin or perfluorocarbons, LEH has several potential advantages:

1. A longer circulation time
2. Reduced vasoactivity
3. The potential to co-encapsulate allosteric modifiers and antioxidants
4. An oxygen diffusivity closer to that of RBCs
5. The ability to be metabolized by the reticuloendothelial system (RES) of the liver and spleen in a similar fashion to RBCs.

Furthermore, most of these properties can be adjusted by altering the properties of the lipid envelope. LEH preparations have been shown to transport oxygen in a variety of animal models.

The reasons that LEH is not more advanced in preclinical and clinical development include inefficiency of production and very high costs. In addition, however, some formulations have activated the complement cascade, and others have been found to cause some of the problems of perfluorocarbon emulsions, which are also particulate in nature. Thus, control and stability of particle size are very important in minimizing toxic side effects.

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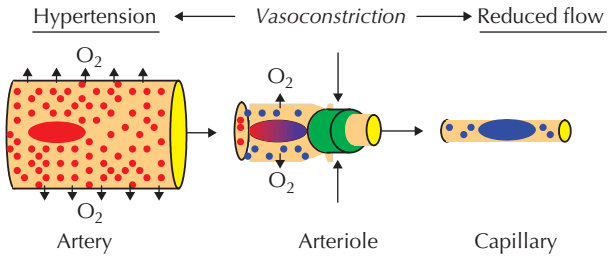

Plate 19 Effect of infusing an early-generation blood substitute with high diffusivity, low viscosity and low O_2 affinity. (From Winslow, 2004). See Fig. 40.1.

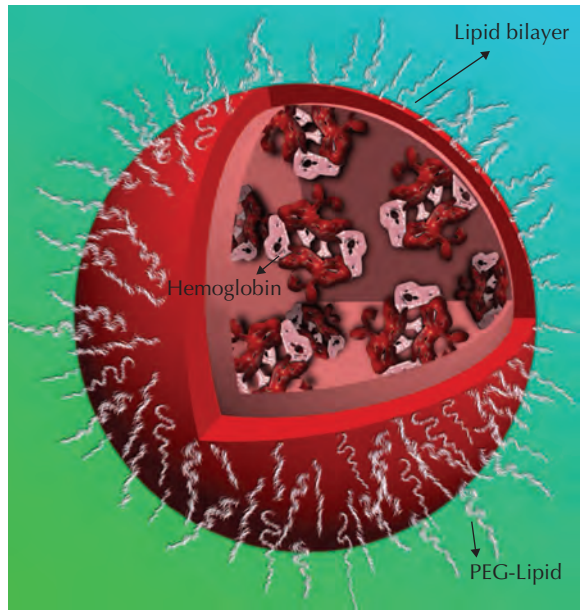

Plate 20 An illustration of liposome-encapsulated hemoglobin. See Fig. 43.1.

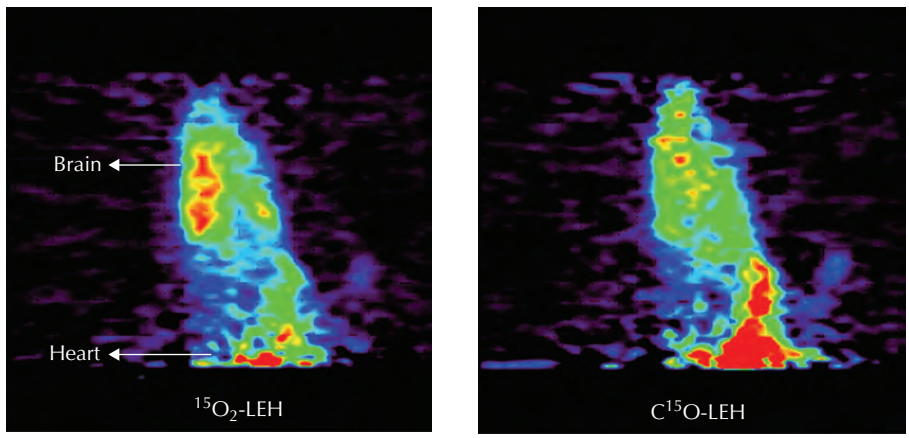

Plate 21 LEH was loaded with O_2 gas or CO (both labeled with O-15) and intravenously injected in a normal rat. Sagittal sections of PET images of rat brain are shown. The image acquisition was initiated with administration of labeled LEH and continued for 7 minutes. LEH releases O_2 to the brain tissue as indicated by the intensity of the brain image, but CO-labeled LEH predominantly shows blood volume in the brain. The degree of oxygen accumulation increases in the following order of color intensity: purple < blue < green < yellow < red. See Fig. 43.4.

Chapter 44

Hemoglobin Vesicles as a Molecular Assembly: Characteristics of Preparation Process and Performances as Artificial Oxygen Carriers

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IMPORTANCE OF CELLULAR STRUCTURE

Physicochemical analysis has revealed that the cellular structure of RBCs may not be effective for the facilitated O_2 releasing and binding of Hb molecules in comparison with a homogeneous Hb solution (Vandegriff and Olson, 1984; Page *et al*., 1998; Sakai *et al*., 2003a); however, nature has selected this cellular structure during its evolution. Historically, Barcroft *et al*. insisted that the reasons for the Hb encapsulation in red blood cells were: (1) a decrease in the high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of the removal of hemoglobin from the blood circulation; and (3) preservation of the chemical environment in the cells such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes (Tsuchida *et al*., 1995). Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs) many side effects of molecular Hb have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$) that may induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow and tissue oxygenation at microcirculatory levels (Goda *et al*., 1998; Sakai *et al*., 2000a), neurological disturbances, and the malfunctioning of the esophageal motor function (Murray *et al*., 1995). These side effects of molecular Hb would imply the importance of the cellular structure.

The pioneering work of the Hb encapsulation was performed by Chang (1991) using a polymer membrane. After Bangham and Horne (1964) had reported that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it was quite reasonable to use such vesicles for the Hb encapsulation. Djordjevici and Miller (1977) prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid etc. The Naval Research Laboratory showed the remarkable progress of LEH (Rudolph

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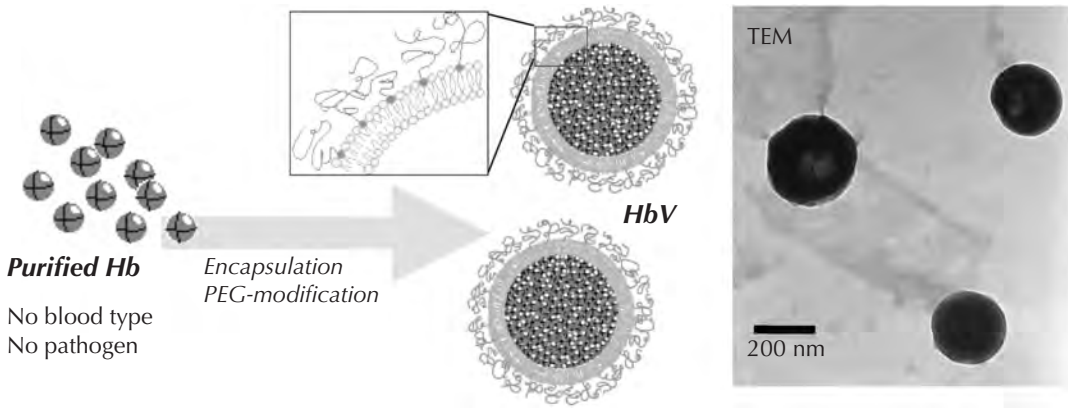

Figure 44.1 Hb vesicles (HbV, diameter *ca*. 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC. One particle contains about 30 000 Hb molecules. The surface of one HbV is modified with about 6000 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles. See color plate 22.

et al., 1991). What we call Hb vesicles (HbV) with a high-efficiency production process and improved properties have been established by Tsuchida's group based on the nano-technologies of molecular assembly and precise analyses of the pharmacological and physiological aspects (Tsuchida, 1998; Figure 44.1). The *in vivo* studies of HbV have revealed O_2 transporting efficiency comparable to that of RBCs (Izumi *et al.*, 1996, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 2004a; Yoshizu *et al.*, 2004), safety in terms of blood compatibility (Ito *et al.*, 2001; Wakamoto *et al.*, 2001), the importance of the particle size and the cellular structure of HbV (Goda *et al.*, 1998; Sakai *et al.*, 2000a), and prompt degradation in the reticuloendothelial system (Sakai *et al.*, 2001, 2004b, 2004c, 2004d), all of which make us confident about advancing to the further development of HbV. The joint collaborative partnership of academia (Waseda and Keio Universities), a biotechnology venture company (Oxygenix, Inc., Tokyo) and a corporation (Nipro Co., Osaka) is aiming for clinical trials of HbV within a few years.

In this chapter we scientifically summarize the characteristics of the preparation process of HbV based on the sciences of molecular assembly to induce their excellent performances. It should be emphasized that the components of HbV, lipids and Hb assemble to form a functional nanoparticle through secondary binding forces (hydrophobic interaction, Coulombic force, hydrogen bond, van der Waals force).

PREPARATION OF HEMOGLOBIN VESICLES

Virus inactivation and removal during hemoglobin purification

The primary advantage of artificial O_2 carriers should be no fear of infectious disease derived from human blood. In Japan, the donated blood is strictly inspected by the nucleic acid amplification test (NAT). However, it is necessary also to introduce procedures to inactivate and remove viruses during the process of Hb purification from outdated RBC in order to guarantee the utmost safety from infection, based on the unforgettable tragedy of HIV transmission due to the distribution of non-pasteurized plasma-derived products. In our purification process, virus inactivation was performed by pasteurization at 60°C for 10 hours – the same conditions for the pasteurization of human serum albumin (Sakai *et al.*, 1993; Fukutomi *et al.*, 2002). This process can be introduced by utilizing the stability of carbonylhemoglobin (HbCO). The thermograms of HbCO indicated a denaturation temperature of 78°C, which is much higher than that for oxyhemoglobin (64°C) (Sakai *et al.*, 2002a).

The virus inactivation efficiency was evaluated by the Hokkaido Red Cross Blood Center (Abe *et al.*, 2001; Huang *et al.*, 2002). The Hb solution spiked with vesicular stomatitis virus (VSV) was treated at 60°C for 1 hour under a CO atmosphere. VSV was inactivated at > 6.0 $\log_{10}$ without metHb

formation and denaturation. Some protein bands other than Hb disappeared on SDS-PAGE and IEF after the heat treatment. During pasteurization, all the other concomitant proteins are denatured and precipitated. As a result, we obtain an ultra-pure Hb solution. This high purity is essential for preventing membrane plugging during the subsequent ultrafiltration process to remove virus. The FDA requires two orthogonal steps of not only virus inactivation but also virus removal.

We tested the ultrafiltration of the HbCO solution to remove viruses with PLANOVA™-35N and -15N (P35N, P15N, Bemberg Microporous Membrane: BMM; Asahi Kasei Co.) (Naito *et al.*, 2002). The virus removal mechanism is by size exclusion through the capillary pores, and a depth filtration. The unit membrane, which has a network structure of capillaries and voids, is accumulated to form 150 layers. P35N and P15N have mean pore sizes of 35 nm and 15 nm, respectively. P35N is suitable for removing envelope-type viruses of which the size ranges from 40 nm to 100 nm, such as HIV, HCV etc., and P15N can be used to remove the non-envelope-type viruses with size of less than 40 nm, such as parvoviruses. The permeation flux and the permeated ratio of the HbCO solution ([Hb] = 5.6 g/dl) through P35N at 13°C were 36 (l/m^2 per hour) and almost 100 (per cent), respectively. Those through P15N were 15 (l/m^2 per hour) and 95 (per cent), respectively. Under the same conditions, a high removal efficiency of a bacteriophage, $\phi \times 174$, ($>$7.7 log) was confirmed. These results indicate that P15N is effective for the process of virus removal from Hb solution. We also confirmed the effectiveness of other virus removal ultrafiltration systems such as Viresolve (Millipore Co.).

The obtained purified HbCO solution can be very effectively concentrated to above 40 g/dl using an ultrafiltration process. After regulation of the electrolyte concentrations, this is supplied for the encapsulation procedure. The ligand of the resulting HbV, CO, is converted to O_2 by illuminating the liquid membrane of the HbV suspension with a visible light under flowing O_2 (Chung *et al.*, 1995).

Other groups have selected the way to preserve the well-organized but relatively unstable enzymatic systems originally present in RBCs, aiming at the prolonged stability of the ferrous state of Hb (Ogata *et al.*, 1997). The enzymatic system can partly be preserved with the compensation of insufficient virus removal or inactivation, but this cannot guarantee the utmost safety of the resulting HBOCs. One advantage of HbV is that any reagent can be co-encapsulated in the vesicles. It has been confirmed that co-encapsulation of the appropriate amount of a reductant (such as glutathione or homocysteine) and active oxygen scavengers (such as catalase) effectively retards the metHb formation (Takeoka *et al.*, 1997, 2002; Sakai *et al.*, 2000a; 2004d; Teramura *et al.*, 2003). However, our recent idea is that the metHb formation may not be a serious problem in the emergency situation because HbV will be infused to bridge to the blood transfusion in a clinical setting.

Encapsulation of concentrated Hb in HbV

The performance of Hb vesicles depends on the weight ratio of Hb to lipid ([Hb]/[lipids]), that is, the ability to carry more Hb with fewer vehicles made of lipids. This value is improved by lowering the number of bilayer membranes (lamellarity) of the vesicle and raising the concentration of Hb in the interior of the vesicle. We studied the optimal conditions for the Hb encapsulation using the extrusion method, and considered the behaviors of the Hb and lipid assemblies as a kind of polymer electrolyte (Takeoka *et al.*, 1993, 1994a, 1994b, 1996).

The maximum ([Hb]/[lipids]) ratio can be obtained at ~pH 7, which relates to the isoelectric point (pI) of Hb. The Hb molecule is negatively charged when the pH is above 7.0, and the electrostatic repulsion between Hb and the negatively charged bilayer membrane results in lower encapsulation efficiency. However, the lower pH enhances the Hb denaturation by too much interaction with the lipid bilayer membrane and metHb formation at a lower pH. Therefore, the physiological pH, 7.0–7.4, is optimal. It was also revealed that the higher ionic strength shields the repulsion between the negatively charged lipid bilayer membranes and increases the lamellarity.

The number of bilayer membranes decreases with increasing the microviscosity (decreased lipid mobility). Multilamellar vesicles are converted to smaller vesicles with a smaller lamellarity during the extrusion procedure. When the membrane fluidity is high, deformation of the vesicles during extrusion occurs more easily even for multilamellar vesicles, resulting in larger lamellarity in the final vesicles. Therefore, the use of lipids with a higher phase transition temperature is preferred. However, these lipids make extrusion more difficult, because a higher shear rate (high

extrusion pressure) is required. Based on this reasoning, mixed lipids contain dipalmitoylphosphatidylcholine (DPPC) as the main component.

Based on the precise analysis of the characterization of the physicochemical properties of the components, the encapsulation efficiency of the Hb solution in a size-regulated phospholipid vesicle has been improved using an extrusion method (Sakai *et al.*, 1996; Sou *et al.*, 2003a). Mixed lipids (DPPC, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[mono-meth oxy poly(ethylene glycol)$_{5000}$]) at a molar ratio of 5 : 5 : 1 : 0.033 were hydrated with a NaOH solution (7.6 mM) to obtain a polydispersed multilamellar vesicle dispersion (50 nm–30 μm in diameter). The polydispersed vesicles were converted to smaller vesicles having an average diameter of ~500 nm and with a relatively narrow size distribution by freeze-thawing at a lipid concentration of 2 g/dl and a cooling rate of −140°C/min. The lyophilized powder of the freeze-thawed vesicles was rehydrated into a concentrated Hb solution (40 g/dl) and retained the size and size distribution of the original vesicles. The resulting vesicle dispersion smoothly permeated through the membrane filters during extrusion. The average permeation rate of the freeze-thawed vesicles was ~30 times faster than that of the simple hydrated vesicles. During the extrusion process, the Hb solution was effectively encapsulated into the reconstructed vesicles with a diameter of 250 ± 20 nm, and the Hb/lipid ratio reached 1.7–1.8. This improvement of the Hb encapsulation procedure is a breakthrough for the scalability for commercialization.

REGULATION OF OXYGEN AFFINITY

The O_2 affinity of purified Hb (expressed as *P*50, the O_2 tension at which Hb is half-saturated with O_2) is about 14 mmHg, and Hb strongly binds O_2 and does not release O_2 at 40 mmHg (the partial pressure of mixed venous blood). Historically, it has been considered that the O_2 affinity should be regulated to a level similar to that of RBC, namely about 25–30 mmHg, using an allosteric effector or by a direct chemical modification of the Hb molecules. Theoretically, this allows sufficient O_2 unloading during blood microcirculation, as can be evaluated by the arteriovenous difference in the levels of O_2 saturation in accordance with an O_2 equilibrium curve. It has been supposed that decreasing the O_2 affinity (increasing *P*50) will result in an increase in the O_2 unloading, which is supported by the result that RBC with a high *P*50 shows an enhanced O_2 release for improved exercise capacity in a mouse model (Shirasawa *et al.*, 2003).

If this theory is correct, the *P*50 of Hb in HbV should be equivalent to that of human red blood cells, i.e., 28 mmHg, or higher. Pyridoxal 5′-phosphate (PLP) is co-encapsulated in HbV as an allosteric effector to regulate *P*50 (Sakai *et al.*, 2000b). The main binding site of PLP is the N-terminal of the α- and β-chains and β-82 Lysine within the β-cleft, which is part of the binding site of natural allosteric effector, 2,3-diphosphoglyceric acid (2,3-DPG). The bound PLP retards the dissociation of the ionic linkage between the β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Thus the O_2 affinity of Hb decreases in the presence of PLP. The *P*50 of HbV can be regulated to 5–150 mmHg by co-encapsulating the appropriate amount of PLP or inositol hexaphosphate as an allosteric effector (Wang *et al.*, 1992). Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was co-encapsulated, and *P*50 was regulated to 18 mmHg. When the molar ratio PLP/Hb was 3/1, *P*50 was regulated to 32 mmHg. The O_2 affinities of HbV can be regulated quite easily without changing the other physical parameters, whereas in the case of the other modified Hb solutions their chemical structures determine their O_2 affinities and thus regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not been yet completely decided; however, the easy regulation of the O_2 affinity may be useful in meeting the requirement for clinical indications such as oxygenation of ischemic tissues (Contaldo *et al.*, 2003).

STORAGE STABILITY

Since Hb autoxidizes to form metHb and loses its O_2-binding ability during storage as well as during blood circulation, the prevention of metHb formation is required. Some groups have reported a method of preserving the deoxygenated Hb in the liquid state (Kerwin *et al.*, 1999), using the well-known intrinsic characteristic of Hb that its oxidation rate in a solution is dependent on the O_2 partial pressure and deoxyHb is essentially not autoxidized at ambient temperature (Levy *et al.*, 1988). For the HbV, not only the inside Hb but also the cellular structure has to be physically

stabilized in order to prevent intervesicular aggregation, fusion and leakage of the encapsulated Hb.

The surface modification of phospholipid vesicles with poly(ethylene glycol) (PEG)-conjugated lipid is a well-known method of prolonging the circulation time of the vesicles *in vivo* for drug delivery systems (Klibanov *et al.*, 1990). For HbV, the surface was also modified with PEG chains to improve the dispersion state of the vesicles when mixed with blood components (Yoshioka, 1991). The PEG-modified HbV has shown an improved blood circulation and tissue oxygenation due to the absence of HbV aggregate formation and viscosity elevation (Sakai *et al.*, 1997, 1998) and prolonged circulation persistence *in vivo* (Sou *et al.*, 2003b). However, little attention has been paid to the ability of the PEG modification for the long-term preservation of vesicles or liposomes in the liquid state. We studied the possibility of the long-term preservation of Hb vesicles by the combination of two technologies – surface modification of HbV with PEG chains, and deoxygenation during storage for 2 years (Sakai *et al.*, 2000c). The samples stored at 4° and 23°C showed a stable dispersion state for 2 years, though the sample stored at 40°C underwent precipitation and decomposition of the vesicular components, a decrease in pH, and 4 per cent leakage of the total Hb after 1 year. The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion due to their steric hindrance. The original metHb content (~3 per cent) before the preservation gradually decreased to less than 1 per cent in all the samples after 1 month due to the presence of homocysteine inside the vesicles, which consumed the residual O_2 (thiol groups in homocysteines reacted with oxygen to generate disulfide and active oxygen species) and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure, and no increase in the metHb formation was observed due to the intrinsic stability of the deoxygenated Hb. These results clearly indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years.

Generally, phospholipid vesicles are regarded as unstable capsules; however, the establishment of this pivotal technology will enhance the application of PEG-modified vesicles in other fields. The long-term preservation of O_2 carriers overcomes the limitation of the blood transfusion system and will contribute to benefiting clinical medicine.

ENDOTOXIN

The production process of HbV has to be guaranteed by a good manufacturing practice (GMP) standard as a biological product regarding the strict regulation of impurities and viral and bacterial contamination. Monitoring the content of the lipopolysaccharide (LPS), known as an endotoxin, a component of the outer membrane of Gram-negative bacteria possessing a large variety of biological influences on numerous mammalian cells and tissues, is strictly required. The US FDA has established a guideline for the human maximal endotoxin dose permissible for parenteral products (5 EU/kg) that may include HBOCs. This limit is based on the endotoxin activity (Endotoxin Unit: EU; 1 EU = 100 pg), and can be measured via the *Limulus amebocyte* lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS (Levin and Bang, 1964). Since the volume of O_2 carriers to be infused for shock resuscitation or acute hemodilution is estimated to be less than 20 ml/kg, the specific endotoxin limits per ml should be 0.25 EU/ml (= I5/20), similar to that for water for injection (0.25 EU/ml).

Bacterial LPS is a gigantic amphiphilic macromolecule, therefore it interacts hydrophobically with protein and biomembranes. Hb strongly interacts with LPS, showing synergistic toxicity. The constituent of endotoxin that causes LAL gelation is a glycophospholipid – designated lipid-A. Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A, such as its LAL gelation activity (Richardson *et al.*, 1983). As a consequence, the researchers who have studied HbV or other phospholipid vesicles for delivering other functional molecules have encountered a problem in measuring the LPS content for the quality control of these materials (Cliff *et al.*, 1995; Harmon *et al.*, 1997).

Considering this background information, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the subsequent LAL assay of the kinetic-turbidimetric gel clotting analysis using a Toxinometer® (Sakai *et al.*, 2004e). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition

that allowed the measurement of LPS higher than 0.1 EU/ml in the HbV suspension.

This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer® is routinely used in our HbV production system. Significant attention is paid to the quality control of HbV for preclinical studies, and all the HbV prepared under sterile conditions showed an LPS content less than 0.2 EU/ml at [Hb] = 10 g/dl. Moreover, the utilization of the histidine-immobilized agarose gel (Pyrosep®) effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/ml. The pretreatment with $C_{12}E_{10}$ would be applicable not only to HbV but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules.

HEMOGLOBIN VESICLES AS OXYGEN CARRIERS *IN VIVO*

The advantages of HbV and other HBOCs are the absence of blood-type antigens and infectious viruses, and stability for long-term storage that outdoes the RBC transfusion. The shorter half-lives of the HBOCs in the bloodstream (2–3 days) limit their use, but they are applicable for a shorter period of use, such as (1) a resuscitative fluid for hemorrhagic shock during an emergency situation for a temporary time or bridging until the packed RBCs are available; (2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage in an elective surgery to avoid or delay allogeneic transfusion; (3) a priming solution for the circuit of an extacorporeal membrane oxygenator (ECMO) (Yamazaki *et al.*, 2004); and (4) other potential indications, e.g. so-called O_2 therapeutics to oxygenate ischemic tissues.

One particle of HbV (diameter ~250 nm) contains about 30 000 Hb molecules. Since HbV acts as a particle in the blood and not as a solute, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires addition of a plasma expander for a large substitution of blood to maintain blood volume. The candidates for plasma expanders are HSA, hydroxyethyl starch, dextran or gelatin, depending on the clinical setting, cost, country concerned and clinicians. Recombinant human serum albumin (rHSA) is the alternative. The absence of any infectious disease from humans is the greatest advantage of rHSA, which will be soon approved for clinical use in Japan. Moreover, there should be no immunological and hematological abnormalities, which are often seen when using dextran and hydroxyethyl starch. Aimed at the application of HbV suspended in a plasma expander for the above indications, HbV was tested in resuscitation from hemorrhagic shock (Sakai *et al.*, 2002b, 2004a; Yoshizu *et al.*, 2004) and extreme hemodilution (Izumi *et al.*, 1997; Kobayashi *et al.*, 1997; Sakai *et al.*, 1997, 1998, 1999) in collaboration with Waseda–Keio and Professor Marcos Intaglietta at UCSD. Moreover, HbV with a high O_2 affinity (low *P*50) suspended in HES or dextran was tested for oxygenation of an ischemic skin flap by Erni *et al.* at the Inselspital University Hospital, Berne (Contaldo *et al.*, 2003; Erni *et al.*, 2003) and the results imply the further application of HbV for other ischemic diseases such as myocardial and brain infarction, and stroke.

SUMMARY

Based on the above establishment of the HbV production system and the potential clinical applications of HbV, significant efforts have been made in the joint collaboration partnership of Waseda–Keio–Oxygenix–Nipro to produce HbV with a facility of GMP standard, and to start preclinical and, finally, clinical trials. Since the combination of recombinant Hb (rHb)-vesicles suspended in recombinant albumin (rHSA) would be the ideal 'artificial red blood cells', this project has recently initiated the next generation HbV (Kai *et al.*, 2004).

EDITOR'S SUMMARY

Liposome encapsulated hemoglobin is a long-sought goal in Japan, where the product is called hemoglobin vesicles (HbV), which distinguishes this product from the one developed primarily in the US, whose designation is LEH. HbV is the result of a long series of studies in which the size of the vesicles, including the number of lipid layers, the surface composition and materials co-encapsulated have been optimized.

HbV is produced by an extrusion process that has commercial potential, although at this time the product has not yet been produced in

quantities sufficient for clinical trials. Sterilization of the hemoglobin, prior to encapsulation, is performed using heat, and antioxidants are co-encapsulated to retard hemoglobin oxidation. Oxygen affinity is regulated to any desired *P*50 by co-encapsulation of allosteric effectors, and this group has contributed important studies on the effect of different *P*50 on oxygen delivery to tissues by HbV. The product is claimed to be stable when stored for up to 2 years.

A commercial effort has been launched in Japan, and it is hoped that HbV could be in human clinical trials within the next few years.

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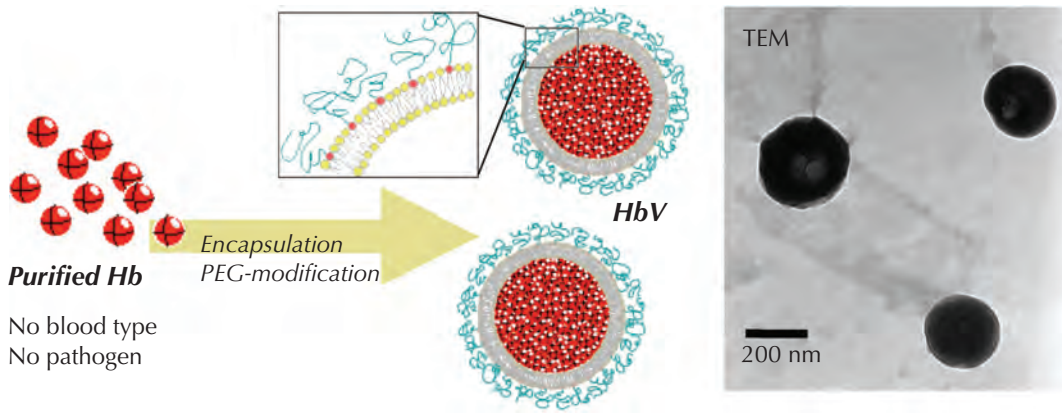

Plate 22 Hb vesicles (HbV, diameter *ca*. 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC. One particle contains about 30 000 Hb molecules. The surface of one HbV is modified with about 6000 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles. See Fig. 44.1.

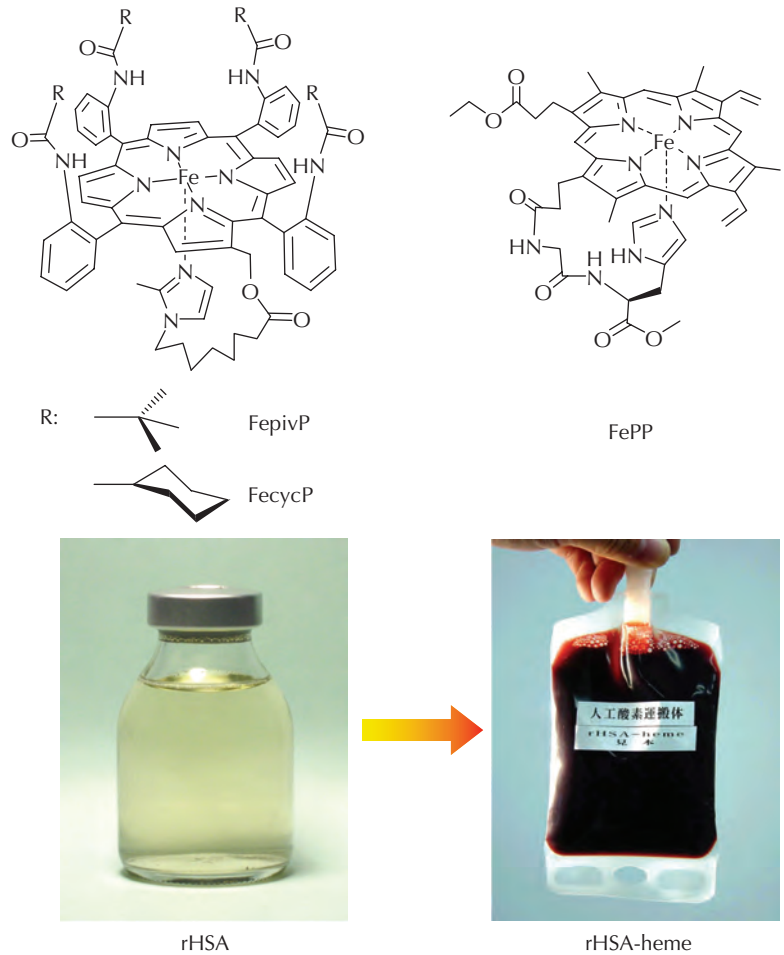

Plate 23 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See Fig 46.1.

Chapter 45

Nanodimension Biodegradable Polymeric Membrane Artificial Red Blood Cells

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INTRODUCTION

The first artificial red blood cells prepared in 1957 by microencapsulation of hemoglobin solution or hemolysate had an oxygen dissociation curve similar to that of red blood cells (Chang, 1957). Further studies resulted in a number of microcapsule membranes including cellulose, silastics,1,6-hexamethylenediamine, crosslinked protein, bilayer phospholipid-cholesterol complexes on crosslinked protein membrane or polymers (Chang, 1964, 1965, 1972). However, even with diameters as small as 1 μm they survived only for a short time in the circulation after intravenous injections. Our finding that sialiac acid in red blood cells is responsible for their long-term survival in the circulation led us to study the effects of changing surface properties of the artificial red blood cells (Chang, 1964, 1965, 1972). This has resulted in significant increases in circulation time, although still not sufficient for actual applications. Djordjevich and Miller (1980) increased the circulation time further by preparing smaller lipid membrane artificial red blood cells with diameter of about 0.2 μm. Further research by a number of groups resulted in hemoglobin lipid vesicles with promising results (Hunt *et al.*, 1985; Farmer *et al.*, 1988; Phillips *et al.*, 1992; Usuba *et al.*, 1992; Tsuchida, 1994). Success in this artificial red blood cell approach towards clinical use means that we are ready to take the next step to further improve on this by preparing biodegradable polymeric hemoglobin nanocapsules that have the following properties:

1. Persistence, with sufficient time in the circulation after infusion
2. Stability in storage
3. Stability after infusion during function as a blood substitute, but biodegradable soon after the completion of action in the body
4. Non-toxicity of the membrane material and its degradation products
5. Presence of important red blood cell enzymes such as superoxide dismutase, catalase, carbonic anhydrase, methemoglobin reductase and others.

We have been using biodegradable polymer, e.g. d,l-polylactic (PLA) acid, for the encapsulation of hemoglobin, enzymes and other biologically active material since 1976 (Chang, 1976). More recently, we have started to prepare hemoglobin nanocapsules of less than 0.2 μm mean diameter using a biodegradable PLA acid membrane and other biodegradable polymers (Chang and Yu, 1992, 1997; Yu and Chang, 1994, 1996). PLA degrades into lactic acid, a normal human metabolite, and finally into water and carbon dioxide. The rate of degradation can be adjusted

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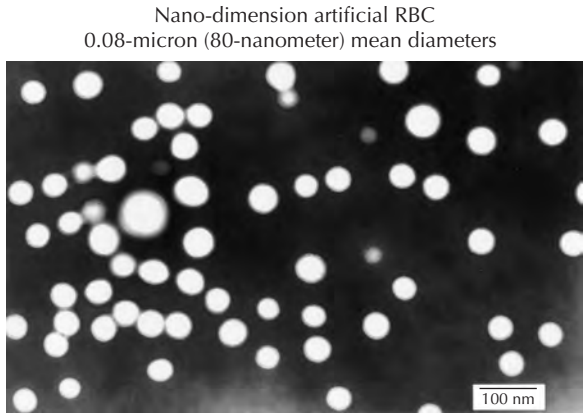

Figure 45.1 Electron micrograph of biodegradable polymer hemoglobin nanocapsules (bar: 100 nm). Reproduced from Yu and Chang (1996), with permission.

by variations in molecular weight, membrane thickness and particle size. Polyesters like PLA and polyglycolic acid have been used extensively since 1982 as surgical implants and sutures with no adverse or toxic effects (Muller, 1991). We also have long-term experience in biodegradable polymer encapsulation using polylactides, having initiated this in 1976 (Chang, 1976; Zhou and Chang, 1987, 1988).

PROPERTIES OF HEMOGLOBIN POLYLACTIDE NANOCAPSULES

Particle size

A typical electron micrograph for biodegradable polymer hemoglobin nanocapsules prepared with PLA is shown in Figure 45.1. They are spherical and homogeneous. Their diameter in the micrograph ranges from 80 to 120 nm, and their membrane thickness is 5–15 nm. The diameter and size distribution of the biodegradable hemoglobin are determined by using the Nicomp Size Analyzer (Model 370), which operates by light scattering. The average particle sizes of biodegradable nanocapsules containing hemoglobin is dependent on the formula used for preparation. Nicomp Sizer Analyzer shows a unimodal distribution for all samples. With different preparation processes and different polymers, the mean diameters of biodegradable hemoglobin nanocapsules can be as low as 74 nm.

Nanocapsule suspensions can be prepared with up to 15 g/dl total hemoglobin concentration of the suspension. The characteristics of the preparation containing 10.97 g/dl of hemoglobin prepared with d,l-PLA are shown in Table 45.1.

Table 45.1 Characteristics of polymer-hemoglobin

Hemoglobin concentration	10.97 g/dl (15 g/dl)
Polymer concentration	1.2 g/dl
Phospholipid concentration	0.6 g/dl
Specific gravity (22°C)	1.0047
*P*50	28 mmHg
Viscosity (37°C)	3.7–3.8 cPs

Oxygen affinity, Hill coefficient and Bohr effect

The oxygen dissociation curve is determined using the TCS Hemoxanalyser (TCS Medical Products Co., USA). The oxygen dissociation curve of the preparation containing 10.97 g/dl bovine hemoglobin is not significantly different from that of free bovine hemoglobin. The 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, the oxygen affinity of polymer membrane containing hemoglobin changed with pH. The Bohr effect is about −0.22 to −0.24. The ability of this approach to prevent methemoglobin concentration will be discussed later in this chapter.

We have carried out analyses to answer the following questions:

1. How much polymer is present in 1 unit of hemoglobin nanocapsule suspension?
2. What will be the effect of the biodegraded product, lactic acid, even though it is a metabolite normally present in the body?

By assuming that all the polymer added is incorporated into the nanocapsule membrane, the maximal amount of polymer per unit of hemoglobin nanocapsule is shown in Figure 45.2. 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 Figure 45.2, is made up mostly of biodegradable polymer. Since polymer is stronger than lipid and is also porous, much less membrane material is required. Polylactic acid is degraded in the body into lactic acid, which is a normal body metabolite. However, hemorrhagic

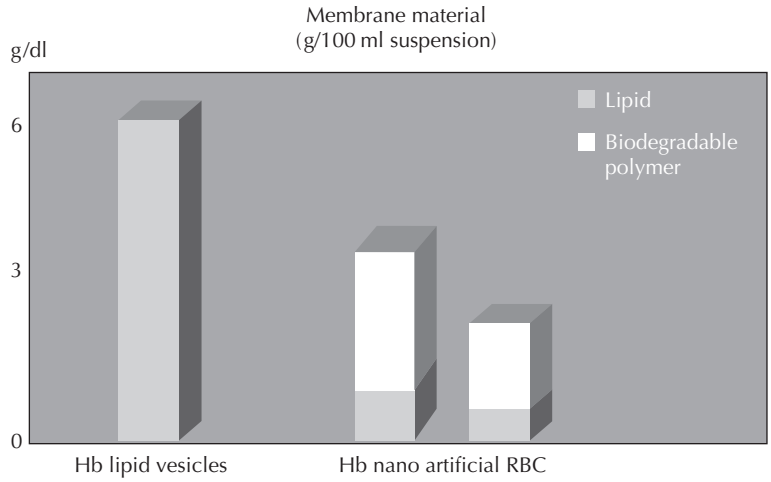

Figure 45.2 Membrane materials of hemoglobin lipid vesicles and hemoglobin nanocapsules. Reproduced from Chang (1997), with permission.

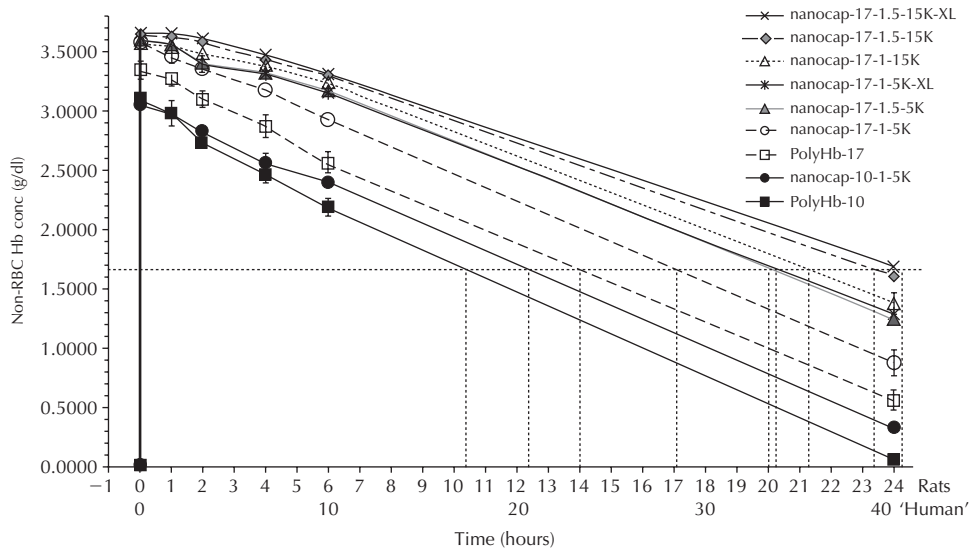

Figure 45.3 Analysis of results. PolyHb-17 is used as the standard for extrapolation of the results obtained for the different types of Hb nanocapsules. The time for PolyHb-17 to reach a non-RBC hemoglobin level of 1.67 g/dl is 14 hours in rats, equivalent to 24 hours in humans. The time to reach this non-RBC hemoglobin concentration of 1.67 g/dl is used to analyze the time for Hb nanocapsules to reach this level. This is then used to calculate the equivalent time for humans. Reproduced from Chang *et al*. (2003), with permission.

shock can result in the production of much lactic acid, and therefore it is important to analyze how much lactic acid is produced in the degradation of polylactic acid hemoglobin nanocapsules. Polylactide is degraded into lactic acid and then water and carbon dioxide. 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/day). The maximal body capacity for breaking down lactic acid is 7080 mEq/day, thus 83 mEq is equal to just 1 per cent 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.

Circulation half-life of hemoglobin nanocapsules

The circulation time of hemoglobin nanocapsules is evaluated using male rats (Figure 45.3).

Cannulation of the femoral artery and vein are carried out, and each rat is injected with one-third of its blood volume by top-loading. The survival time of the nanocapsules in circulation is then followed. Polylactic acid membrane hemoglobin nanocapsules are removed rapidly from the circulation. Incorporation of PEG markedly increased the circulation time of these hemoglobin nanocapsules to a clinically useful duration (Chang *et al.*, 2002, 2003). Details, including the exact circulation time in hours, are discussed below.

PEG-PLA COPOLYMER Hb NANOCAPSULES

Figure 45.3 shows that 30 per cent top-load using preparations with glutaraldehyde crosslinked polyhemoglobin10 g/dl in rats resulted in a maximal non-RBC Hb conc. of 3.35 g/dl, falling to half this (1.67 g/dl) in 14 hours. The molecular weight distribution of this polyhemoglobin prepared in this laboratory is similar to that of those now in Phase III clinical trials. From here on, PolyHb (17 : 1) is used as the basis for comparison with all nanocapsule preparations, including the time taken for the circulating non-RBC Hb of different preparations to reach 1.67 g/dl. Individual or combinations of four factors are used to prepare different types of nanocapsules. These are studied in regard to the maximal systemic Hb attained and the time of maintaining a systemic non-RBC Hb level equal to that of polyHb (17 : 1), 1.67 g/dl (Chang *et al.*, 2003, 2004). The ratio 17 : 1 refers to the molar concentration ratio of glutaraldehyde and hemoglobin used in the preparation of the polyhemoglobin.

The four factors are as follows:

1. The degree of polymerized Hb. Polyhemoglobin is used instead of stroma-free hemoglobin so that if there is any leakage after infusion there will not be adverse effects due to stroma-free hemoglobin; furthermore, polyhemoglobin can continue to function even if a small amount leaks out
2. Effects of higher molecular weight PLA
3. Effects of concentrations of the PEG-PLA copolymer
4. Crosslinking of the newly formed PEG-PLA Hb nanocapsules.

Analysis of the results of these four factors as shown below will allow us to assess which factor is more important and also the cost-effectiveness of the different approaches and combinations of the different approaches.

Effects of molecular weight distribution of polyHb used in the PEG-PLA nanocapsules

A 30 per cent top-load using Hb nanocapsules containing polyHb (10 : 1 is the molar concentration ratio of glutaraldehyde and hemoglobin used in the preparation of the polyhemoglobin) results in a maximal non-RBC Hb level of only 3.05 g/dl (SD = 0.03). The non-RBC Hb falls to 1.67 g/dl in 12.3 hours in rats (Figure 45.3). Calculations based on body weight, blood volume, plasma volume and dilution factors show that the maximal non-RBC hemoglobin concentration for Hb nanocapsules should have been at least 3.6 g/dl, rather than only 3.05 g/dl as for these Hb nanocapsules. This seems to show that a significant amount (about 16 per cent) of the infused Hb nanocapsules has been removed nearly immediately on infusion. Thus the next step is to try to prevent this.

We therefore designed a more refined method to improve the degree of polymerization of polyhemoglobin to reduce markedly the amount of tetrameric hemoglobin (PolyHb 17 : 1). This was then used for nanoencapsulation. As shown in Figure 45.3, 2 minutes after infusion the maximal non-RBC Hb was 3.58 g/dl (SD = 0.04). This is significantly higher than the 3.05 g/dl (SD = 0.04) for the earlier Hb nanocapsules, and also approaches the maximal possible initial non-RBC Hb concentration. Furthermore, it took 17.1 hrs in rats for the non-RBC Hb level to fall to 1.67 g/dl. This very significant increase was further improved in the following stepwise incremental design of Hb nanocapsules until we reached a maximal concentration of 3.66 g/dl (SD = 0.03) and 24.2 hours to fall to the level of 1.67 g/dl in rats.

Effects of higher concentrations of PEG-PLA (polyethelene-glyco-polylactide) copolymer combined with polyHb(17 : 1)

Using the same method as above with PolyHb (17 : 1) but with a 1.5-fold higher PEG-PLA concentration resulted in a thicker membrane with better membrane stability. This gave a further increase in circulation time (see Figure 45.3). Thus, 2 minutes after infusion, the maximal non-RBC Hb was 3.60 g/dl (SD = 0.01). Furthermore, it took 20.0 hours in rats for the non-RBC Hb level to fall to the level of 1.67 g/dl.

We also used a two-fold higher concentration of PEG-PLA polymer to further improve the stability of the membrane. However, the Hb nanocapsules formed this way tended to aggregate and therefore this was not tested in animal studies.

Effects of higher molecular weight PLA for the PEG-PLA copolymer

We looked at the use of a higher molecular weight PLA to increase the stability of the Hb nanocapsule membrane. For this we replaced the 5K PLA with a 15K PLA to form the PEG-PLA copolymer. This also very significantly increases the circulation time of the preparation as compared to that prepared using the method in (1) (see Figure 45.3). Thus 2 minutes after infusion, the maximal non-RBC Hb was 3.57 g/dl (SD = 0.05). The rate of the disappearance was also much slower, but what is more important is that it took 21.2 hours in rats for the non-RBC Hb level to fall to 1.67 g/dl.

Higher PEG-PLA concentration combined with higher molecular weight PLA

We next looked at combining the use of a higher molecular weight PLA (15K) with a 1.5 times higher concentration of the polymer. This therefore provided a combination of the following three factors:

1. Use of polyhemoglobin with a low percentage of crosslinked tetrameric hemoglobin
2. Use of a 1.5-fold concentration of the PLA-co-PEG copolymer
3. Use of a higher molecular weight PLA (15K).

This resulted in a further significant improvement. Two minutes after infusion, the maximal non-RBC Hb was 3.6458 g/dl (SD = 0.02). The rate of the disappearance was also much slower, but what is more important is that it took 23.3 hours in rats for the non-RBC Hb level to fall to the level of 1.67 g/dl (see Figure 45.3).

Effect of crosslinking the newly formed Hb nanocapsules

The method using polyHb (17 : 1) was modified by adding glutaraldehyde to the Hb nanocapsules suspension after they were formed. This approach was used earlier to stabilize both the larger Hb microcapsules and the protein inside (Chang, 1971). The polymerization was stopped by adding 2 M of lysine (at a molar ratio of lysine/hemoglobin = 100 : 1) after 24 hours. This approach also increased the circulation time to the same degree as when using the 1.5-times concentration of the polymer. Thus, two minutes after infusion the maximal non-RBC Hb was higher at 3.60 g/dl (SD = 0.01). The rate of the disappearance was also much slower, but what is more important is that it took 20.3 hours in rats for the non-RBC Hb level to fall to 1.67 g/dl (see Figure 45.3).

Effects of combination of all four factors to prepare Hb nanocapsules

Finally, we combined all the above four factors as follows:

1. Use of a polyhemoglobin (17 : 1) with low percentage of single crosslinked tetrameric hemoglobin
2. Use of a 1.5-fold concentration of the PLA-co-PEG copolymer
3. Use of a higher molecular weight PLA (15K)
4. Crosslinking of the newly formed Hb nanocapsules with glutaraldehyde.

Two minutes after infusion, the maximal non-RBC Hb was higher at 3.6583 g/dl (SD = 0.03) (see Figure 45.3). The rate of the disappearance was also slower, but what is more important is that it took 24.2 hours in rats for the non-RBC Hb level to fall to 1.67 g/dl.

Analysis of results

B shows the effects of infusing the same total amount of hemoglobin of different types of polyhemoglobin and hemoglobin nanocapsules containing the same total amount of hemoglobin: (1) The maximal non-red blood cell hemoglobin reached and (2) the time for which this amount can maintain a non-red blood cell hemoglobin concentration of at least 1.67 g/dl. This is not an analysis of the half-life or the kinetics of removal. These show clearly that 2 minutes after infusion, the maximal Hb concentrations attained with the better Hb nanocapsules are at the maximal levels possible. After a 30 per cent blood volume top-load using Hb concentration of 10 g/dl, the best polyHb-17 (prepared using a glutaraldehyde: hemoglobin ration of 17 : 1) can only attain a maximal Hb concentration of 3.35 g/dl. The best PEG-PLA Hb nanocapsules, on the other hand, can reach a maximal Hb concentration of 3.60 g/dl. This

cannot be explained by polyHb having greater colloid osmotic pressure than Hb inside nanocapsules, resulting in some hemodilution. After all, in those PEG-PLA Hb nanocapsules that are removed more rapidly than PolyHb-17, e.g. nanocap-10-1-5K, the maximal Hb reached for nanocap-10-1-5K is lower than that of PolyHb-17 (see Figure 45.3). The more likely explanation is that the best PEG-PLA Hb nanocapsules are still in the circulation when the first samples are taken 2 minutes after infusion. For nanocap-10-1-5K and PolyHb, a small fraction must have been removed within 2 minutes after infusion.

After a 30 per cent blood volume top-load using Hb concentration of 10 g/dl, the best polyHb can maintain a systemic hemoglobin level reaching 1.67 g/dl after 14 hours in rats (equivalent to about 24 hours in humans). In the case of the best Hb nanocapsules, a similar top-loading can maintain a much longer systemic Hb level, reaching 1.67 g/dl after 24.2 hours in rats (see Figure 45.3). If we use the rat results in this study for polyHb of 14 hours and its clinical equivalent of about 24 hours, we might calculate and extrapolate this as follows. For the best PEG-PLA Hb nanocapsules with the ability to maintain a systemic Hb level reaching 1.67 g/dl after 24.2 hours, it is likely equivalent to about 41.5 hours in human (see Figure 45.3).

Thus, the equivalent functional circulation time in humans after 30 per cent top-loading using the best PEG-PLA Hb nanocapsules could be 41.5 hours when compared to 24 hours for glutaraldehyde crosslinked polyHb. This is a very significant and important increase that would allow longer function after infusion and thus decrease the need for donor blood and further increase the avoidance of donor blood. The Hb nanocapsules are likely to have an even higher circulation time in human compared to PolyHb. This is because in studies on nanocapsules for drug delivery, it has been found that the reticuloendothelial systems in rats are much more efficient at removing particulates like nanocapsules when compared with primates or humans. Thus a circulation half-time of 18 hours in rats is equivalent to about 2.6 days in primates and humans. A further advantage of PEG-PLA nano-encapsulation of polyHb is that even if PolyHb leaked slowly out of nanocapsules after infusion it would continue to act and, unlike stroma-free Hb, would not cause adverse effects. Even in donor red blood cells there is some hemolysis, especially if they are stored longer. Indeed, PEG-PLA nanocapsules could be a useful carrier for other modified Hb, including recombinant Hb and PEG-conjugated Hb, to prolong their circulation time, for inclusion of enzyme systems and to avoid direct external exposure.

ENZYMES AND MULTIENZYMES

Artificial cells have been prepared to include multienzyme systems with cofactor recycling (Chang, 1985). A number of enzymes normally present in red blood cells can be encapsulated within these biodegradable polymer hemoglobin nanocapsules (Chang *et al.*, 2002, 2003). This may be important in, for example, stabilizing 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 shown that this can convert methemoglobin to hemoglobin. Nanocapsules may improve on the problem relating to the methemoglobin reductase system in lipid vesicles 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 Figure 45.4. External glucose can diffuse into the nanocapsules, while products of the reaction can diffuse out and therefore do not accumulate in the nanocapsules to inhibit the reaction. These studies are discussed in further detail below.

PREVENTION OF METHEMOGLOBIN FORMATION

By increasing the circulation time, we have significantly increased the *in vivo* functioning of the hemoglobin in the nanocapsules. However, with an increase in circulation in the body at 37°C, there would be a steady increase in methemoglobin. Oxidation of hemoglobin to methemoglobin inside red blood cells is prevented by the enzyme systems of the red blood cells.

Hb nanocapsules containing metHb reductase system

Hb nanocapsules are prepared that contain all the enzymes of the red blood cells (Figure 45.4).

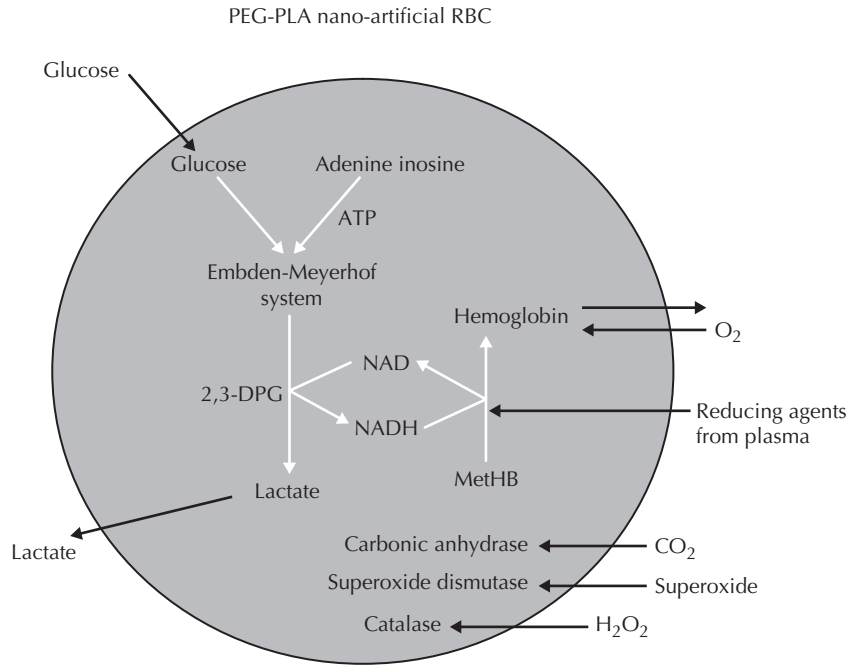

Figure 45.4 Nanodimension artificial red blood cells containing red blood cell enzymes including methemoglobin reductase system, carbonic anhydrase, catalase and superoxide dismutase. Methemoglobin formation can be reduced by either the more complicated methemoglobin reductase system or by plasma reducing agents diffusing into the nanocapsules. Reproduced from Chang *et al*. (2003), with permission.

This is done by extracting all the enzymes and hemoglobin from red blood cells (Chang *et al*., 2002, 2003). We prepared Hb nanocapsules with a higher methemoglobin level of 7 per cent. We then incubated these at 37°C and followed the changes in the percentage of methemoglobin (metHb) as follows:

1. When suspended in Ringer lactate containing 100 mg/dl glucose, metHb in the Hb nanocapsules increased by a total of 2.5 per cent in 6 hours
2. When suspended in lactated Ringer's solution containing 100 mg/dl glucose and 0.02 mM NADH, as glucose and NADH entered the nanocapsules to start the multienzyme reaction, instead of increasing, metHb was decreased by 1.5 per cent in 5 hours. This result is very exciting because it shows that we only need to encapsulate fresh red blood cell contents with the normal amount of methemoglobin reductase system. This way, 100 mg glucose (available as blood glucose) and 0.02 mM NADH in the suspending medium not only prevents metHb formation, but can also convert preformed metHb back to Hb. By further optimization of the NADH concentration, this can be increased further.

Unlike NADH, the larger cofactor NADPH cannot permeate across the nanocapsules, and thus it can be enclosed inside the nanocapsules. This avoids the need to supply external cofactor and allows the reaction to take place as in RBCs.

Hb nanocapsules permeable to reducing factors from plasma

There are reducing agents in the plasma that prevent methemoglobin formation. Examples include ascorbic acid and glutathione. Unlike LEH or HbV, Hb nanocapsules are permeable to these and other plasma-reducing molecules. We suspended Hb nanocapsules in solutions of ascorbic acid, glutathione or methylene blue, and found that the Hb nanocapsule membrane is permeable to all these factors. The Hb in the nanocapsules will therefore be exposed to reducing factors in the circulating plasma that prevent the formation of metHb. This is an additional means of preventing the formation of methemoglobin that can be used to form a simpler system.

Other red blood cell enzymes

Other red blood cell enzymes present in the red blood cell extract, like carbonic anhydrase, catalase and superoxide dismutase, are also included in the PEG-PLA Hb nanocapsules. In addition, higher concentrations of RBC enzymes such as catalase and superoxide dismutase can be included, along with other non-RBC enzymes.

IMPLICATIONS FOR BLOOD SUBSTITUTE DEVELOPMENT

Just as success in crosslinked hemoglobin has stimulated research into next-generation crosslinked hemoglobin, this is also the case in encapsulated hemoglobin. Thus, success in hemoglobin lipid vesicles has encouraged us to look into the next step toward a further generation of encapsulated hemoglobin. We are only at the early stage of this research. In the meantime, it is important to complete the development of the hemoglobin lipid vesicles for clinical trial. It is expected that the first-generation encapsulated hemoglobin will be in routine clinical use before the next-generation hemoglobin nanocapsules will be ready for clinical testing. At present our 21-day long-term studies show that these biodegradable hemoglobin nanocapsules do not have any adverse effects on biochemistry or enzymes.

EDITOR'S SUMMARY

Encapsulation of hemoglobin in lipid vesicles has been possible for over 40 years. On the surface, it would appear that such hemoglobin–lipid vesicles would be more like human red cells than chemically modified hemoglobin. However, there have been several problems with this approach, including low encapsulation efficiency, ready oxidation of hemoglobin, and adherence of hemoglobin to the outside of the vesicles – not to mention the very high cost and complexity of the production process. In this chapter, Dr Chang has outlined a new approach that might address some of these problems. This involves the use of polymerized lactic acid instead of lipids, a material that is biodegradeable and therefore could reduce the toxicity associated with massively overwhelming the reticuloendothelial system. The hemoglobin is polymerized in order to achieve a high *P*50 and reduced renal toxicity if released from the capsules. Vesicles have been prepared with co-encapsulation of reducing substances and enzyme systems normally found in the interior of red cells. Thus the experimental system can be used for a reductive approach to studying how the red blood cell functions with regard to maintenance of its flexibility, reduction of methemoglobin and ultimately its flexibility. While the production of a product that might enter clinical trials in humans would seem to be on the relatively distant horizon, the experimental methods allow the study of transport mechanisms, control of redox cycling of iron, the effects of reactive oxygen species, and the type of surface modification that can be manipulated to influence the intravascular retention time. Thus, this is a very useful research tool that might lead to the better understanding of human red blood cells in addition to providing clues for a next-generation blood substitute.

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Chapter 46

Albumin-Heme: A Synthetic Heme-Based Oxygen Carrier

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INTRODUCTION

The risk of transmission of viral illness by transfused blood has become extremely low and the transfusion of donor blood is currently a routine procedure. However, this level of safety has been achieved at great cost, and hepatitis virus or unknown pathogens cannot be completely excluded by the NAT system. Furthermore, the transfusion of donor blood requires cross-matching and compatibility tests to avoid a hemolytic reaction in the recipient, and the purified red blood cells (RBC) need to be stored in the refrigerator at 4°C. These requirements limit the availability of blood in a disaster or emergency. Against this background, several types of hemoglobin (Hb)-based oxygen carriers (HBOCs) have been studied as potential RBC substitutes or O_2 therapeutic reagents (Chang, 1997; Tsuchida, 1998; Winslow, 1999; Squires, 2002; Greenburg and Kim, 2004). Unfortunately, these materials do not fulfill all the requirements of blood replacement compositions. The first concern is the source of human Hb, which is limited by the availability of outdated human blood. Animal blood raises concerns about the transmission of animal pathogens, as Hb products potentially carry risks due to the biological origin of the raw materials. The second problem of the HBOCs (i.e., modified Hb) is the high colloid osmotic pressure (Keipert and Chang, 1988) and its vasoconstriction effect (Schultz *et al.*, 1993; Abassi *et al.*, 1997; Moisan *et al.*, 1998). About 50 per cent of the products in advanced clinical trials still increase blood pressure and decrease cardiac output (Squires, 2002). The precise mechanism of this hypertension is controversial, but many researchers suspect that the Hb molecules penetrate the vascular endothelium and capture the endothelial-derived relaxing factor (EDRF), namely NO. Others believe that the excessive delivery of oxygen to arteriolar vascular walls induces autoregulatory vasoconstriction (Guyton *et al.*, 1964; Tsai *et al.*, 1995; Rohlfs *et al.*, 1998; Winslow, 2000).

RATIONALE FOR ALBUMIN-HEME

In our circulatory system, free hemin (iron(III) complex of protoporphyrin IX dissociated from metHb) is captured by hemopexin, which is a unique protein having an extremely high binding constant of hemin ($K > 10^{12}\,M^{-1}$) (Tolosano and Altruda, 2002). Crystal structure analysis of the hemopexin–hemin complex has revealed that the hemin is tightly bound by double histidine

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coordinations to the central ferric ion and multiple hydrogen bondings with the amino acid residue (Paoli *et al.*, 1999). Nevertheless, the concentration of hemopexin in the plasma is rather low ($<17\,\mu M$) and human serum albumin (HSA) may provide a reserve binding capacity of hemin in various conditions, for instance trauma, inflammation, hemolysis etc. In fact, HSA binds hemin with a relatively high affinity ($K > 10^8 M^{-1}$) (Adams and Berman, 1980). If HSA can transport O_2 like Hb, it would be of extreme medical importance not only as a blood replacement composition but also as an O_2 therapeutic reagent.

We have found that a series of super-structured heme derivatives with a covalently linked proximal-base were incorporated into HSA, and the obtained red-colored albumin–heme hybrids (Figure 46.1) can reversibly bind and release O_2 under physiological conditions in the same manner as Hb and myoglobin (Mb) (Komatsu *et al.*, 1999, 2000, 2001a, 2002; Tsuchida *et al.*, 1999; Nakagawa *et al.*, 2004). Since recombinant HSA (rHSA) is manufactured on a large scale by yeast expression, the rHSA–heme hybrid has become entirely synthetic hemoprotein and absolutely free of infectious pathogens. Our recent animal experiments demonstrated that rHSA-heme actually works as an oxygen-carrying plasma protein in the bloodstream (Tsuchida *et al.*, 2000; Komatsu *et al.*, 2004). Although the NO-binding affinity of rHSA-heme is higher than that of Hb (Komatsu *et al.*, 2001b), it does not induce an unfavorable vasopressor effect at all

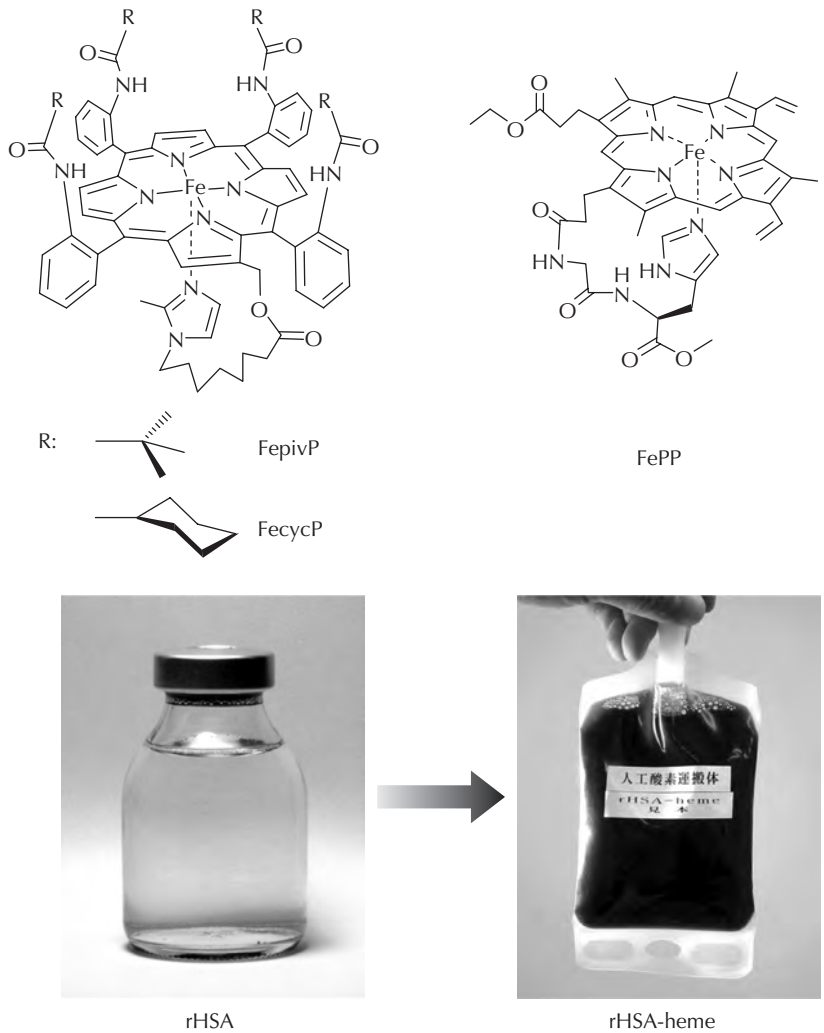

Figure 46.1 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See color plate 23.

(Tuschida *et al.*, 2003). We suspect that the electrostatic repulsion between the albumin surface and glomerular basement membrane around the endothelial cell retards the rapid leakage of the rHSA-heme molecule and quick scavenging of NO. The albumin-heme is now recognized to be a promising material for a new class of RBC substitutes. In this chapter, we describe the O_2-transporting efficacy and preclinical safety of this synthetic heme-based O_2-carrier.

OXYGEN BINDING AND PHYSICOCHEMICAL CHARACTERISTICS

From the 30 super-structured heme compounds that were all synthesized by the authors, we found that oxygenated rHSA-FecycP showed a high stability against the autoxidation; its half-time against the ferric form *in vitro* (9 h at 37°C) was close to that of the native Mb (Komatsu *et al.*, 2002). We selected rHSA-FecycP with a similar *P*50 value (34 mmHg at 37°C) to that of RBCs as the most suitable material for an artificial O_2-carrier. The physicochemical characteristics and shelf-life of the rHSA-heme solution ([rHSA], 5 g/dl; heme/rHSA, 4 (mol/mol); isoelectric point, 4.8; COP, 18 mmHg; viscosity, 1.1–1.2 cPs; shelf-life greater than 2 years) had already been reported elsewhere (Komatsu *et al.*, 1999, 2002; Tsuchida *et al.*, 2002).

BLOOD COMPATIBILITY *IN VITRO*

The viscosity of the rHSA-heme solution (1.2 cPs at a high shear rate of 230 s^{-1}) was much lower than that of whole blood (4.0 cPs) and exhibited Newtonian type shear rate dependence just like rHSA itself. After mixing the rHSA-heme solution into whole blood at 10–44 per cent of the volume, the heme concentration in the plasma phase remained constant for 6 hours at 37°C, and no significant time dependence was observed in the numbers of RBCs, white blood cells and platelets (Huang *et al.*, 2003). The microscopic observations clearly showed that the shapes of the RBC had not been deformed during the measurement period. These results suggest that the rHSA-heme has no effect on the morphology of the blood cell components *in vitro*. With respect to the blood coagulation parameters (prothrombin time and activated partial thromboplastin time), the coexistence of rHSA-heme has only a negligibly small influence. Moreover, it was also shown that the rHSA-heme solution has no influence on the complement factors (CH50, SC5b-9) and platelet activation. Although more functional assay is necessary to establish firmly the biocompatibility of rHSA-heme with whole blood, we can conclude that it has a good compatibility with blood cells.

IN VIVO EFFECTS

Blood pressure effects

The administration of extracellular HBOCs often elicits an acute increase in blood pressure by vasoconstriction. At the beginning of this study, our concern was that the small rHSA-heme molecules (8×3 nm) injected into the blood vessels would be eliminated from the circulation, and contribute to the significant consumption of NO in the interstitial space between the endothelium and vascular smooth muscle. In fact, rHSA-heme strongly binds NO; the NO-binding affinity ($P50^{NO} = 1.8 \times 10^{-8}$ mmHg) is nine-fold higher than that of the Hb, and is high enough to react with 1 μM NO in the wall of the vasculator (Komatsu *et al.*, 2001b). In order to clarify the hemodynamic behavior after the administration of this entirely synthetic O_2-carrying hemoprotein, we tested a top-load dose of the rHSA-heme solution in anesthetized rats (Tsuchida *et al.*, 2003). Contrary to our expectations, only a negligibly small change in the mean arterial pressure (MAP) was observed after the administration of the rHSA-heme solution (5 g/dl, 300 mg/kg; Figure 46.2a). If anything, the difference from the baseline (ΔMAP) slowly decreased to -6.8 ± 3.4 mmHg within 20 minutes and remained constant during the monitoring period. The response was completely the same as that observed following infusion with an equivalent volume of rHSA (5 g/dl) in this experimental set-up. In contrast, the administration of extracellular Hb solution elicited an acute increase in blood pressure (ΔMAP 16 ± 1.9 mmHg), which followed a graduated decrease throughout the 60-minute period of observation (Tsuchida *et al.*, 2003). Why does rHSA-heme not induce the hypertension? The answer probably lies in the negatively charged molecular surface of the albumin vehicle. One of the unique characteristics of serum albumin is its low permeability through the muscle capillary

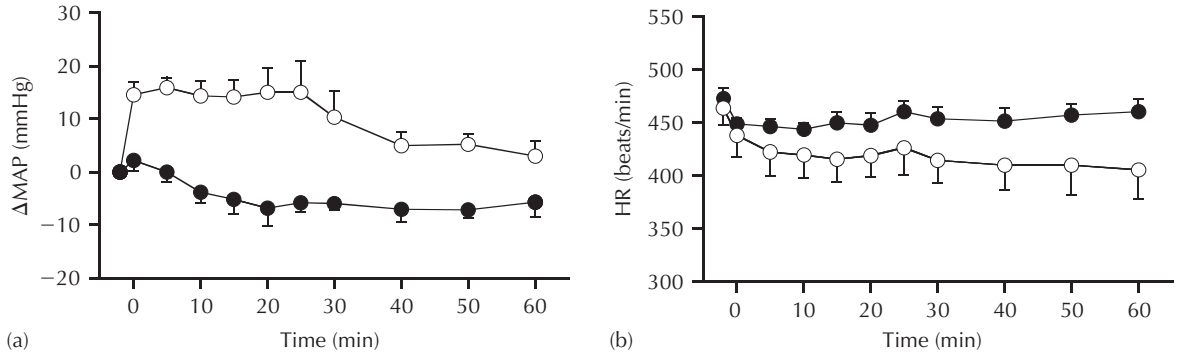

Figure 46.2 Changes of (a) MAP and (b) HR in anesthetized rats before and after infusion of rHSA-heme solution (n = 5) (●, rHSA-heme group; ○, Hb group). MAP is represented as change from the basal value (ΔMAP) just before the infusion with mean ± SEM (n = 5) (basal value is 90.1 ± 3.0 mmHg). HR was shown as mean ± SEM (n = 5) (Tsuchida, 2003).

pore, which is less than 1/100 that for Hb due to the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells. In the blood vessels, rHSA-heme presumably circulates for a longer time compared to Hb without extravasation. The heart rate (HR) responses after the rHSA-heme injection were also negligibly small (Figure 46.2b). Visualization of the intestinal microcirculatory changes clearly showed that the widths of the venule and arteriole are fairly constant (Tsuchida *et al.*, 2003).

Hemodilution

The physiological responses to a 30 per cent exchange transfusion with rHSA-heme solution after 70 per cent hemodilution with 5 g/dl rHSA were investigated using anesthetized rats (Komatsu *et al.*, 2004). First, the isovolemic 70 per cent hemodilution was carried out using 5-g/dl rHSA solution. The blood withdrawal via the common carotid artery (2 ml) and the rHSA infusion from the femoral vein (2 ml) (each 1 ml/min) were repeated for nine cycles until Hct was reduced to 13.6 per cent (32 per cent of the initial Hct value of 42.6 per cent). After 10 minutes, a 30 per cent volume of the circulatory blood was withdrawn, producing a severe hemorrhagic shock state. The same volume of the samples was then intravenously injected. As negative or positive control groups, the rats were infused with the 5-g/dl rHSA solution (rHSA group) or the shed rat blood ([heme] = 5.3 mM, whole blood group). The circulation parameters, blood parameters, renal cortical PO_2 (PtO_2(R)) and muscle tissue PO_2 (PtO_2(M)) were carefully monitored for 60 minutes after the injection.

Following administration of the 5-g/dl rHSA solution, the MAP, HR, respiration rate, PtO_2(R), PtO_2(M), arterial blood O_2 pressure (PaO_2), venous blood O_2 pressure (PvO_2), and arterial blood CO_2-pressure ($PaCO_2$) did not recover, leading to death within 32 minutes (Figure 46.3). By contrast, the infusion of whole blood improved these values, except for PtO_2(M), to their initial levels. In the rHSA-heme group, the animals survived over 60 minutes after the infusion, and the HR, respiration rate, PtO_2(R) and PvO_2 showed similar recoveries to those as observed in the whole blood group (Komatsu *et al.*, 2004). MAP, PtO_2(M), PaO_2, pH and PCO_2 also significantly recovered. We are certain that the albumin-heme solution has the potential to resuscitate hemorrhagic shock, stabilize the blood circulation, and transport oxygen throughout the body.

PRECLINICAL SAFETY

In order to evaluate the preclinical safety of this synthetic O_2 carrier, we performed a 20 per cent exchange transfusion with rHSA-heme into anesthetized rats and measured the time courses of the circulation parameters (MAP, HR, respiration rate) and blood parameters (PaO_2, PvO_2, pH, blood cell numbers) for 6 hours, which is adequate time to identify acute toxicity (Huang *et al.*, 2004a). After stabilization of the animals' condition, the

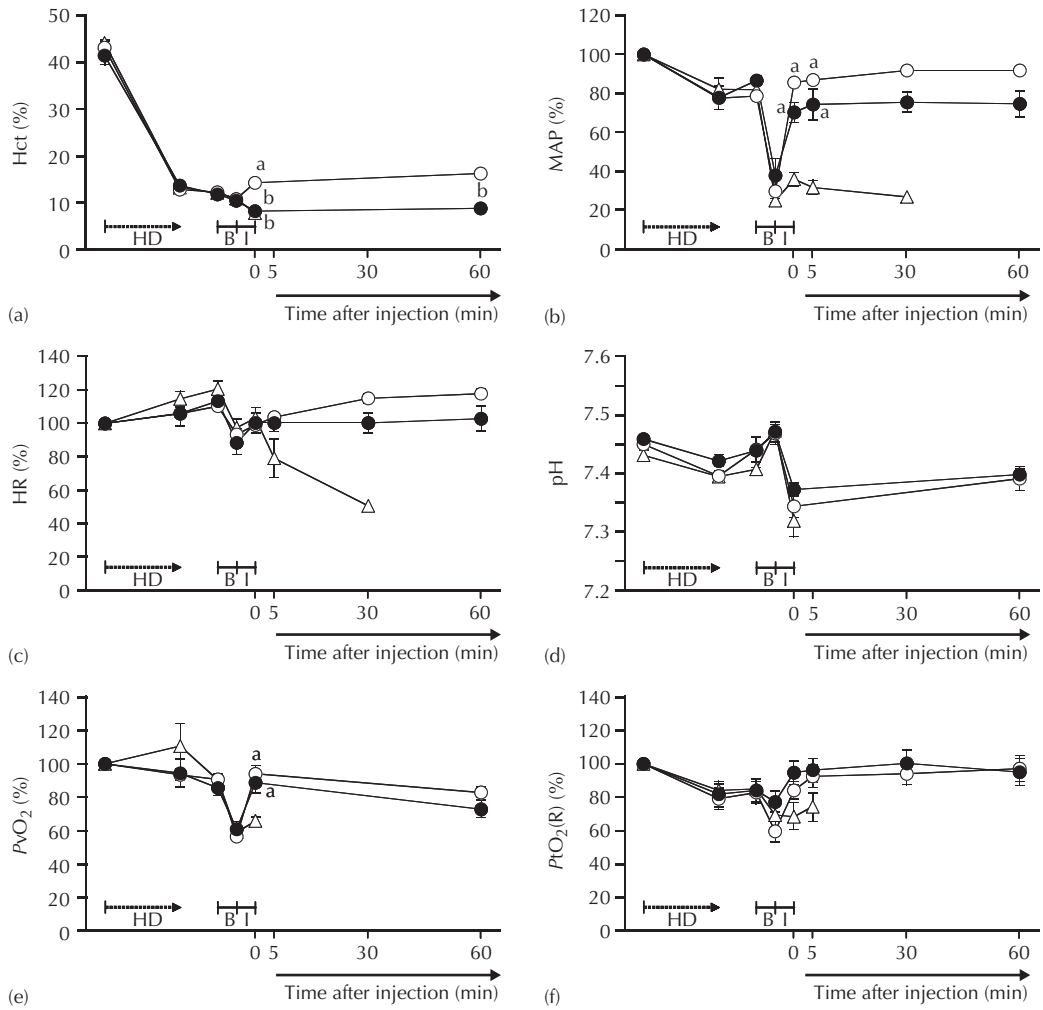

Figure 46.3 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PvO_2 and (f) PtO_2(R) in anesthetized rats after 70 per cent hemodilution with rHSA and 30 per cent exchange transfusion with rHSA-heme solution (n = 6) (●, rHSA-heme group; ○, whole blood group; △, rHSA group). MAP, HR, PvO_2 and PtO_2(R) are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH were shown as mean ± SEM. HD, hemodilution; B, bleeding; I, sample injection. [a]$P < 0.05$ versus rHSA group. [b]$P < 0.05$ versus whole blood group (Komatsu, 2004).

20 per cent exchange transfusion was performed by 1 ml blood withdrawal via the common carotid artery and 1 ml rHSA-heme infusion from the femoral vein (each 1 ml/min) with four repeating cycles.

The appearance of all the animals showed absolutely no change for 6 hours after the exchange transfusion. The physiological responses of the blood circulation, gas equilibria and blood cell numbers in the rHSA-heme group were almost the same as those of the control group (surgery treatments without infusion) and rHSA groups (Figure 46.4; Huang *et al.*, 2004a). MAP and HR did remain constant after the injection of the rHSA-heme, suggesting again that the albumin-based O_2 carrier does not induce the vasoconstriction. It is also noteworthy that autoxidation of the ferrous rHSA-heme to the ferric state was retarded in the bloodstream; the half-time of the oxygenated rHSA-heme *in vivo* was approximately four-fold longer than that *in vitro* (Tsuchida *et al.*, 2000). It was found that autoxidated rHSA-hemin was certainly reduced in the whole blood suspension. A physiological concentration of ascorbic acid continuously provided by RBC probably re-reduces the ferric hemin, leading to the apparent long lifetime of the oxygenated species.

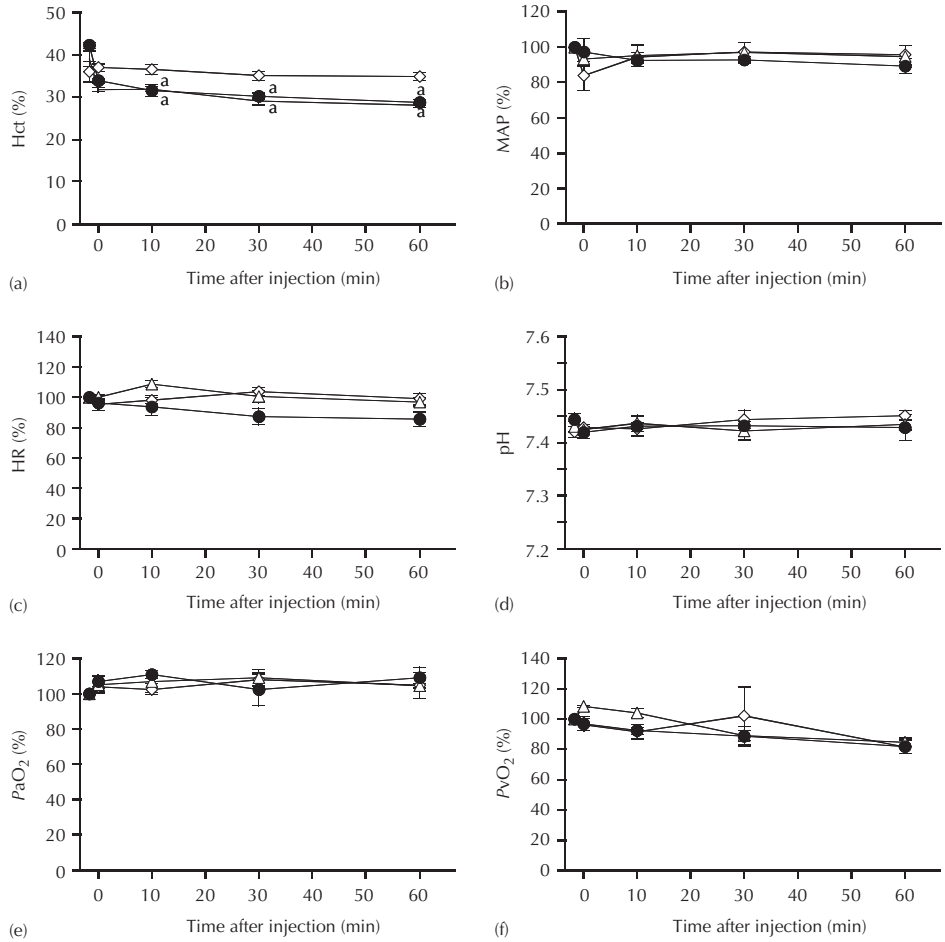

Figure 46.4 Time courses of (a) Hct, (b) MAP, (c) HR, (d) pH, (e) PaO_2 and (f) PvO_2 in anesthetized rats after 20 per cent exchange transfusion with rHSA-heme or rHSA solution (n = 6) (◇, control group (only surgery treatments without infusion); △, rHSA group; ■, rHSA-heme group). MAP, HR, PaO_2 and PvO_2 are represented as percentage ratios of the basal values with mean ± SEM. Hct, HR and pH are shown as mean ± SEM. (Huang, 2004a).

Furthermore, 20 per cent exchange transfusions with rHSA-heme into anesthetized rats were followed by blood biochemical tests of the withdrawn plasma and histopathology observations of the vital organs for 7 days (Huang *et al.*, 2004b).

In the albumin-heme group, a total of 30 analytes showed almost the same values, by blood biochemical tests, as those observed in the reference rHSA group, implying that there was no significant toxicity caused by the exchange transfusion with rHSA-heme (Huang *et al.*, 2004b). Histopathology observations implied that the administration of rHSA-heme did not produce any negative side effects on the vital organs. All these results showed the preclinical safety of the rHSA-heme solution.

FUTURE RESEARCH

As described in this chapter, results have shown O_2-transporting efficacy and initial clinical safety of the rHSA-heme solution that allow us to undergo further advanced preclinical testing of this synthetic O_2 carrying plasma protein. Exchange transfusion with rHSA-heme into beagles is now under investigation.

Furthermore, rHSA-heme as a monomolecular O_2 carrier was tested for its ability to increase O_2 tension in the hypoxia of the solid tumor rat model. By the direct administration of the rHSA-heme solution (10 ml/kg) into the ascites hepatoma LY80 tumor on the femur, the O_2 tension of the hypoxic region immediately increased

to 3.45 ± 1.43 mmHg, which corresponds to a 2.4-fold increase compared to that of the baseline value (Kobayashi *et al.*, 2003). These high O_2 levels continued for 300 s after the infusion. While more research is required to consider how rHSA-heme behaves in the tumor blood vessel and is related to the increase in the O_2 partial pressure, the present results obviously indicate that rHSA-heme led to increased O_2 release in the hypoxic region in the solid tumor. Experiments regarding combined treatment with the rHSA-heme administration and radiation therapy are currently underway.

ACKNOWLEDGMENTS

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EDITOR'S SUMMARY

Albumin-heme is a unique product – an oxygen transporter that is totally synthetic. By this approach, human serum albumin is produced in a recombinant yeast system, and then synthetic heme is coordinated to its surface. Up to 8 heme groups per molecule have been incorporated so far.

Albumin-heme has been prepared to have a *P*50 similar to that of red blood cells, but the oxygen binding is not cooperative. It avidly binds NO, but is not hypertensive in preliminary animal tests. It appears to be as stable with regard to oxidation as native human hemoglobin. A number of early preclinical tests have been performed, including biocompatibility and effects on coagulation, and no significant toxic effects have been noted.

While it is still early in the development of albumin-heme as a therapeutic agent for use in humans, and the cost of production is likely to be high, it is an intriguing product that could find use in specialized applications such as oxygenating tumors to increase the effects of radio- or chemotherapy.

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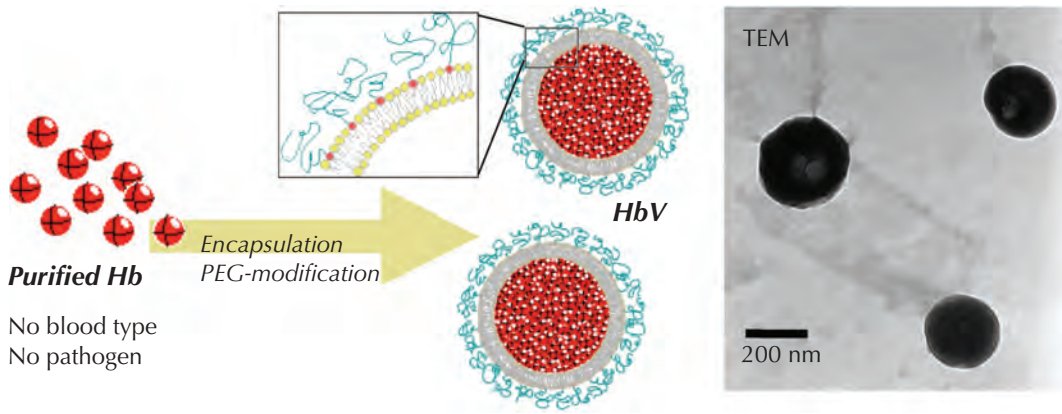

Plate 22 Hb vesicles (HbV, diameter *ca*. 250 nm) are prepared from ultra-pure Hb obtained from outdated RBC. One particle contains about 30 000 Hb molecules. The surface of one HbV is modified with about 6000 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. The transmission electron micrograph (TEM) clearly demonstrates the well-regulated particle size and high Hb content within the vesicles. See Fig. 44.1.

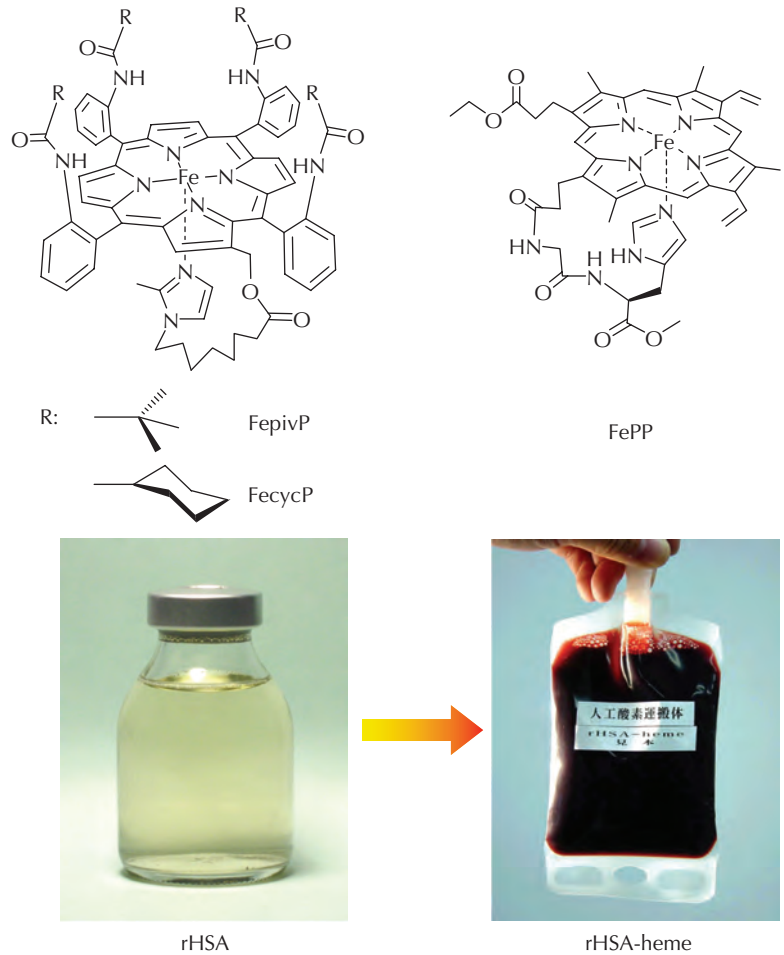

Plate 23 Super-structured heme derivatives for the albumin-heme hybrids and the red-colored rHSA-heme solution ([rHSA] = 5 g/dl). See Fig 46.1.

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