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人工血液

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ARTIFICIAL BLOOD

Vol. 5 No. 2 June, 1997

会　告

1.第9回北海道輸血シンポジウム

メインテーマ：「輸血代替療法の展望」

日　時：　平成9年7月4日（金）・　5日（土）

場　所：　北海道大学学術交流会館（札幌市北区北8条西5丁目）

事務局：　北海道赤十字血液センター（Tel:011-613-6121, Fax:011-613-4131）

※入場無料

※日本輸血学会認定医単位更新の対象，北海道医師会生涯教育講座3単位認定対象となります.

プログラム

第1日目：7月4日（金）　9:30～17:30

開会の辞　　関口　定美（北海道赤十字血液センター）

I. 基調講演

1)　司会　　湯浅　晋治（順天堂大学医学部）

演者　　関口　定美（北海道赤十字血液センター）

2)　司会　　高折　益彦（川崎医科大学）

演者　　畔　　政和（国立循環器病センター）

3)　司会　　伊藤　和彦（京都大学医学部）

演者　　土田　英俊（早稲田大学理工学総合研究センター）

II. 人工赤血球

司会　　小林　紘一（慶応義塾大学医学部）

演者　　高折　益彦（川崎医科大学）

Burhop, KE (Baxter Healthcare)

谷口　直之（大阪大学医学部）

III. サイトカインによる輸血代替療法

司会　　新津洋司郎（札幌医科大学）

演者　　平嶋　邦猛（埼玉医科大学）

宮崎　洋　（キリンビール株式会社医薬探索研究所）

池淵　研二（北海道赤十字血液センター）

第2日目：7月5日（土）　9:30～17:00

IV. 人工血小板およびその他

司会　　高橋　恒夫（東京大学医科学研究所）

演者　　池田　康夫（慶應義塾大学医学部）

丹羽　光一（北海道赤十字血液センター）

守沢　和也（日本油脂株式会社筑波研究所）

V. 造血幹細胞の応用

司会　中畑　龍俊（東京大学医科学研究所）

演者　岡本真一郎（慶應義塾大学医学部）

小川眞紀雄（サウスカロライナ医科大学）

中畑　龍俊（東京大学医科学研究所）

平山　文也（北海道赤十字血液センター）

VI. 総合討論「輸血代替療法への期待」

司会　清水　勝　（東京女子医科大学）

池田　康夫（慶應義塾大学医学部）

閉会の辞　関口　定美（北海道赤十字血液センター）

2．平成9年度　日本血液代替物学会　定期総会

日　時：　平成9年9月7日（日）17:00～17:30

場　所：　リーガロイヤルホテル早稲田

議　事：　1）平成8年度事業報告承認の件

2）平成8年度収支決算承認の件

3）平成9年度事業計画および収支予算決議の件

4）役員選任の件

5）7-ISBSの件

6）その他

※参加できない正会員の方は必ず同封の委任状を事務局までご提出下さい.

3．第7回　血液代替物国際会議（7-ISBS）
（兼　第4回日本血液代替物学会年次大会）

日　時：　平成9年9月8日（月）9:00～10日（水）14:00

会　場：　早稲田大学　国際会議場　〒169-50 新宿区西早稲田1-6-1

大会長：　土田 英俊　早稲田大学

関口 定美　北海道赤十字血液センター

T.M.S. Chang　マッギル大学

主　催：　日本血液代替物学会，早稲田大学，

ISABI (International Society for Artificial Cells, Blood Substitutes, and Immobilization Biotechnology)

後　援：　文部省，厚生省，日本赤十字社，科学技術振興事業団，日本輸血学会，

日本血液事業学会，日本医師会，日本獣医師会，日本薬学会，日本人工臓器学会，

生産開発科学研究所

参加要領：ハガキに「第7回 血液代替物国際会議」と標記し，参加者氏名，勤務先，連絡先（住所，電話，FAX番号）を明記し，下記事務局宛にお申し込み下さい．資料を送付いたします．口頭発表は既に締切りましたが，ポスター発表は7月15日まで受け付けております．

論文投稿：本会議は年次大会を兼ねております．発表された演題の「人工血液」への投稿を歓迎しております．会期中会場受付に提出いただきますと，学会発表の場での査読を予定しておりますので，きわめて速報性高く掲載されます．また，日本語での投稿も可能です．投稿規定は「人工血液」の裏表紙に掲載されておりますので参考にして下さい．

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第7回血液代替物国際会議事務局
Tel: 03-3200-2669, 03-5286-3120
Fax: 03-3205-4740
e-mail: 7isbs@mn.waseda.ac.jp

PROGRAM OVERVIEW (Tentative)

Date	Sep 7 (Sun)	Sep 8 (Mon)		Sep 9 (Tue)			Sept 10 (Wed)		
	Rihga Royal Hotel	1F Ibuka Hall	Conf Rm 3	1F Ibuka Hall	Conf Rm 1	Conf Rm 3	3F Ibuka Hall	Conf Rm 1	Conf Rm 3
8:00	Registration	Registration	Chamber Music, Opening Remark	Registration			Registration		
9:00					Session 3	Session 5		Session 7	Session 5
10:00			PL		Coffee Break	Coffee Break	PS-3 Discussion	Coffee Break	Coffee Break
11:00			Coffee Break PS-1 Discussion		Session 4	Session 6		Session 5	Session 8
12:00			Session 1						
13:00			Lunch Break / Lunch PS-1 Discussion					Session 9	
14:00			Session 1						
15:00			Coffee Break PS-2 Discussion					Closing Remark	
16:00			Session 1						
17:00			Supper Break / Supper PS-2 Discussion						
18:00	Mixer Party		Welcome Party						
19:00			Session 2						
20:00									
21:00									

PL: Plenary Lecture
PS: Poster Session

セッションおよび主な招聘講演者

Plenary Lectures

E. Tsuchida (Waseda U.)
R. M. Winslow (UCSD)

Session 1　Clinical Trials of Blood Substitutes

S. A. Gould (Northfield Laboratories Inc.)
J. DeAngelo (Apex Bioscience Inc.)
R. G. L. Shorr (Enzon Inc.)
C. J. C. Hsia (Synzyme Technologies, Inc.)
R. F. Caspari (Somatogen Inc.)
T. H. Schmitz (Baxter Healthcare Co.)
P. E. Keipert (Alliance Pharmaceutical Co.)

Round Table Discussion: The Target and Assessment of Clinical Tests

A. G. Greenburg (The Miriam Hospital)

Session 2　Blood Service

S. Sekiguchi (Hokkaido Red Cross Blood Center)
T. C. Drees (Sanguine Co.)

Session 3　Microcirculation

K. Messmer (Munich U.)
M. Intaglietta (UCSD)

Session 4　Development in Encapsulated Hemoglobin

T. M. S. Chang (McGill U.)
K. Kobayashi (Keio U.)
A. S. Rudolph (Naval Research Lab.)

Session 5　New Materials and Methods

J. C. Fratantoni (C.L.McIntosh & Ass.)
T. C. Fisher (U. Southern California)
J. D. Hellums (Rice U.)
R. Barbucci (U. Siena)
T. Yonetani (U. Pennsylvania)
D. T-B Shih (Taipei Med. College)
E. Bucci (U. Maryland)
J. Ning (Hemosol Inc.)

Session 6　Perfluorochemicals

J. Riess (Nice U.)
K. C. Lowe (Nottingham U.)

Session 7 Blood Programs in East Asia and Oceania

J. Wang (Chinese Acad. Med. Sci.)
R. O'Charoen (Thai Red Cross Soc.)
K. S. Han (Seoul National U.)
M. Wikanta (Indonesian Red Cross)
P. M. Deninngton (Red Cross Tran. Srv. Queensland)

Session 8 Toxicity and Safety Evaluation

A. Alayash (FDA)
J. C. Bakker (the Netherlands Red Cross)

Session 9 Platelet Substitutes

B. M. Alving (Washington Hospital Center)
Y. Ikeda (Keio U.)

大会長挨拶

酸素を運ぶ赤血球代替物，すなわち酸素輸液を実現させたいという研究は我が国でも既に昭和30年代に着手されました．その後紆余曲折を経て，独特の切り口による研究が継続されて参りましたが，国家的方策に基づいた強力な開発がなされて来た米国と対比しますと，散発的な状況にありました．然し，この分野の重要性緊急性に鑑み有志の総意が集合され，臨床医学から理工学にわたる関連研究者を集めた日本血液代替物学会の設立（1993年）となり，会員各位の御尽力を得てようやく設立4年目を迎えることができました．

本学会では血液代替物全般を対象として，内外情報の収集と提供，年次大会と研究発表会の開催，会誌「人工血液」の発刊，血液代替物に関する評価基準の設定など，明日への展開に繋ぐ活動を積極的に推進しております．また，この分野に対する日本血液代替物学会の貢献と活動，更に我が国臨床医の強い関心が認められまして，北米大陸を離れた企画として初めて今回東京が第7回国際会議開催地に選ばれました．会員の皆様の御協力の賜物であり，大変喜ばしいことに存じております．

米国での研究グループは一部の新しい代替物について既に臨床第3相試験を終了しており，本会でも注目しております．この国際会議ではまず，北米で第一線にある臨床と基礎の研究者に最先端の成果を報告願い，我が国での研究成果も交えて討論を行う企画を設定致しました．また，血液事業の世界的な現状や代替物の適応目標も浮き彫りする企画としております．更には，血液代替物の効能を微小循環から評価し，副作用や代謝系など未解決点も明らかにしたいと考えました．我が国での開発例の特徴でもありますカプセル型ヘモグロビンも充分な議論ができるようにし，血小板代替物，その他など，新しい試みの将来展望も討論の場を設けてあります．

本会議ではまさに「臨床に登場する血液代替物」が，我が国で初めてお目見えを果す機会でもありますので，初日には特別に同時通訳も準備致し各位の御討論と御理解の一助と致しました．会員の皆様に御参集いただき積極的な御討議を期待致しております．尚，本国際会議は第4回日本血液代替物学会年次大会（1997年）と開催を兼ねております．

HEMOGLOBIN-BASED BLOOD SUBSTITUTES AND MECHANISMS OF TOXICITY

Abdu I. Alayash, Ph.D., Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892 USA

Chemical and genetic manipulations of stroma-free hemoglobin have been used to produce physiologically viable oxygen-carrying blood substitutes. The nature of some of these modifications, may however, play a significant role in influencing the level and extent of tissue damage due to hemoglobin-mediated pro-oxidative reactions. In an endothelial cell model of toxicity, chemically modified hemoglobins and simpler recombinant myoglobin prototypes with key distal amino acid substitutions were evaluated. Data from these studies will be presented to describe some of the structural dynamics that govern hemoprotein's redox reactions. These finding may ultimately help in the design of safer second generation hemoglobin-based blood substitutes.

RECENT DEVELOPMENTS AND FUTURE PERSPECTIVES OF PLATELET SUBSTITUTES

Barbara M. Alving and Chitra Krishnamurti
The Washington Cancer Institute at the Washington Hospital Center and Walter Reed Army Institute of Research, Washington, D.C., U.S.A.

Multiple factors are driving the development of frozen platelets and platelet substitutes. These include the short, five-day shelf-life of platelets, the increasing requirement for platelet products in patients who are undergoing intensive chemotherapy or allogeneic stem cell transplants, the concern for the viral and bacterial contamination of platelets, and the necessity for ease of storage and transport in field medicine. The products that are undergoing evaluation include platelets that have been stored at 4° C in the presence of cytochalasin B and EGTA-AM to prevent activation, platelets frozen in 5% or 6% solutions of DMSO, platelets that have been freeze-dried after treatment with formaldehyde, platelet membranes for infusion, and albumin microspheres with covalently-bound fibrinogen on the surface. Issues concerning the preparation of platelet products include demonstration of viral inactivation, definition of biologic activity in preclinical testing, and choice of appropriate clinical trials to evaluate efficacy. One approach to viral inactivation utilizes psoralen compounds and long-wavelength ultraviolet radiation. Examples of *in vitro* assays of platelet function include assessment of morphology scores and osmotic reversal reactions, measurement of activation by flow cytometry, and measurement of platelet force development in response to thrombin. Preclinical *in vivo* studies have assessed the correction of the bleeding time in thrombocytopenic animal models and correction of surgically-induced bleeding in dogs treated with aspirin. The most difficult aspect of platelet development is the demonstration of clinical efficacy. The purpose of some preparations, especially those with limited circulation times, will be to control active bleeding in thrombocytopenia, and appropriate clinical settings will need to be defined. Currently, only one product has undergone phase 2 clinical testing (Infusible Platelet Membranes™). Due to the difficulty of demonstrating clinical efficacy, strategies will be needed to define surrogate markers of hemostasis. Furthermore, evidence that such preparations do not induce clincal thrombosis will be required. The wide range of platelet products now under development will stimulate researchers, clinicians and regulatory officials to define the potential efficacy of such products in improving hemostasis in patients with thrombocytopenia or platelet dysfunction (endogenous or drug-induced) who are at high risk for bleeding or who are bleeding actively.

CHARACTERIZATION OF A 260 kDa TETRAHEMOGLOBIN OBTAINED BY CHEMICAL CROSSLINKING OF A CYSTEINE-CONTAINING DIHEMOGLOBIN

David A. Marquardt, Janet K. Epp, Jon Vincelette, Michael Suniga, Michael P. Doyle, and Spencer J. Anthony-Cahill
Research and Development, Somatogen Inc., 2545 Central Avenue, Boulder CO 80301

A recombinant dihemoglobin on which a surface Lys has been changed to Cys (SGE2812) has been expressed in high yield in *E. coli*. SGE2812 has been purified and characterized in vitro by SEC, C4 RP HPLC, PMB thiol titration, equilibrium oxygen binding, ESMS and light scattering. The purified dihemoglobin was then crosslinked to form tetrahemoglobin using the homobifunctional Cys-specific crosslinker bismaleimidohexane. Purified tetrahemoglobin was administered to conscious rats and mean arterial pressure (MAP) and heart rate were measured for 90 minutes. The change in MAP was substantially lower in rats that received tetrahemoglobin compared to rats that received the monohemoglobin rHb1.1. A mild bradycardia, consistent with the observed MAP elevation, was observed after administration of tetrahemoglobin. These results support earlier findings at Somatogen (B. Kerwin *et al.*; S. Trimble *et al.*, J. Davidson *et al.*) which suggest that a larger hemoglobin molecule attenuates the hemodynamic response observed in conscious animals upon administration of rHb1.1.

DESIGN, CONSTRUCTION AND CHARACTERIZATION OF SELF-ASSEMBLING OLIGOMERIC RECOMBINANT HEMOGLOBINS

Janet K. Epp, Charles D. Kang, Jon Vincelette, Jeffrey S. Davidson, Björn Albrecht, Evie Verderber, Michael Suniga, Michael P. Doyle, and Spencer J. Anthony-Cahill
Research and Development, Somatogen Inc., 2545 Central Avenue, Boulder CO 80301

Fusion of extrinsic oligomerizing domains to the dialpha chain of the "monohemoglobin" rHb1.1 leads to the expression of hemoglobin oligomers in *E. coli*. The following domains have been fused to the C-terminus of dialpha globin: (1) coiled-coil dimerizing domain from yeast GCN4, (2) tetramerizing domain from human p53, (3) pentamerizing domain from rat COMP, (4) tetramerizing domain from bacteriophage P22 Mnt, (5) a tetramerizing coiled-coil based on GCN4. In addition, dimerizing domains from GCN4 and P22 Arc have been inserted between dialpha globins in dihemoglobin constructs developed at Somatogen in order to obtain tetrahemoglobins. Most of these constructs have been expressed, purified and characterized in vitro by SEC, C4 RP HPLC, equilibrium oxygen binding, ESMS and light scattering. Three of these oligomeric fusion proteins (diα-p53, diα-COMP and diα-GCN4-diα) have been administered to conscious rats and mean arterial pressure (MAP) and heart rate were measured for 90 minutes. Hemodynamic responses to these molecules have been complex. In general, the initial pressor responses observed with the oligo-rHbs were substantially diminished versus the pressor response seen with the rHb1.1 control group. Significant tachycardia was observed in the rats following administration of oligo-rHbs. All three oligo rHbs showed longer circulating halflives than rHb1.1. These results demonstrate that fusion of extrinsic oligomerizing domains to globin gene sequence does yield higher MW rHbs.

EXPRESSION, PURIFICATION AND CHARACTERIZATION OF A RECOMBINANT DIHEMOGLOBIN MOLECULE

David A. Marquardt, Jeffrey S. Davidson, Michael P. Doyle, Jon Vincelette, Jeffry Nichols†, Jacqueline F. Aitken and Spencer J. Anthony-Cahill
Research and Development, Somatogen Inc., 2545 Central Avenue, Boulder CO 80301
† Department of Biochemistry, Rice University, Houston Texas.

A recombinant dihemoglobin (SGE946) which is made up of a single chain tetra-α globin and four β globins has been expressed in *E. coli*. The sequence of the single chain tetra-α is: αI-Gly-αII-SerGlyGlySerGlyGlySer-αIII-Gly-αIV. SGE946 has been purified and characterized in vitro by SEC, equilibrium oxygen binding, analytical ultracentrifugation, and ESMS. Two other constructs with fourteen and sixteen amino acid linkers between αII and αIII have also been expressed and characterized. The observed values of P_{50} and n_{max} for all three dihemoglobins are slightly lower than those observed for the recombinant hemoglobin rHb1.1 (a "monohemoglobin" comprised of two β globins and an αI-Gly-αII dialpha globin chain). Changes in blood pressure and heart rate following adminstration of dihemoglobin were monitored in conscious rats. The pressor response was reduced in rats that received dihemoglobin compared to those that received rHb1.1. These observations demonstrate that functional hemoglobin oligomers with desirable *in vivo* properties can be directly expressed as soluble proteins in bacteria. Recombinant DNA technology may provide the ability to design and produce multiple hemoglobin therapeutics with functional properties tailored to specific clinical applications.

A STRATEGY FOR VALIDATION OF ULTRAFILTRATION FOR VIRUS REMOVAL: POINTS TO CONSIDER

M. Azari Ph.D., A. Ebeling, K. Ogle Ph.D., S. Guzder, J. Catarello, J. Paine, T. Camacho, R. Sarajari, J. Herren, T. Estep Ph.D., K. Rohn, T. Marshall, K. Burhop Ph.D., Blood Substitutes Program, Baxter Healthcare Corporation, Round Lake, Illinois, USA 60073

To enhance product safety and comply with virus safety guidelines, a virus removal step -- tangential flow ultrafiltration -- was designated in the production process of Diaspirin Crosslinked Hemoglobin (DCLHb) to supplement the validated, primary step for heat inactivation of viruses. A multi-part strategy was devised to validate the ultrafiltration process for virus removal: defining and designing a practical and relevant scaled-down test system; defining the operating parameters; devising and validating two complementary, non-destructive, quantitative integrity tests that correlate with virus retention of the filter; and formal validation of the filters for virus removal.

An ultrafiltration test system was designed and optimized for validation studies at 1:290 the scale of the system used in the full-scale manufacturing process. This scaled-down test system was designed to use the smallest amount of hemolysate required to operate the system and still be monitored accurately for key process parameters. The test solution volume was chosen so that high virus titers could be obtained after inoculating the solution with a practical, obtainable quantity of virus.

The key process parameters used for operation of the test system in the validation studies were the worst practical case, relative to virus retention. These parameters, which were determined by performing a series of preliminary studies, included hemolysate concentration in the test solution, volume of test solution per unit area of the filter, transmembrane pressure of the filtration system, crossflow of the filtration system, and pore size of the filter. Marker virus models Bacteriophage ϕX174 and Encephalomyocarditis Virus were used to determine these worst case parameters.

The integrity tests [wet air diffusion rate (ADR) and PVP K-90 retention determination] were correlated with the virus retention of the ultrafiltration system using marker virus models Bacteriophage ϕX174, Encephalomyocarditis Virus, and Porcine Parvovirus. These tests were used to determine minimum acceptance criteria for the filter to remove viruses. The ADR determination test was selected to confirm the integrity of the filters after each use, as the filters are used multiple times. The ADR range correlating with mammalian virus removal was identified and will be utilized to determine the point at which the usage of filters will be terminated.

Once the scale, operating parameters and integrity test issues were resolved, formal validation was performed in which the scaled-down ultrafiltration system was challenged, in at least duplicate, with several relevant model viruses: Bovine Viral Diarrhea Virus, Pseudorabies Virus, Human Immunodeficiency Virus, Porcine Parvovirus and Hepatitis A Virus. The formal validation studies performed using the worst practical conditions, scale and model viruses, in conjunction with the primary, validated heat treatment step, demonstrated significant virus removal for all five viruses evaluated.

VALIDATION OF VIRUS INACTIVATION DURING THE CROSSLINKING AND HEAT TREATMENT STEPS USED IN THE PRODUCTION OF DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb)

M. Azari Ph.D., A. Ebeling, J. Catarello, K. Burhop Ph.D., T. Camacho, T. Estep Ph.D., S. Guzder, T. Marshall, K. Rohn, R. Sarajari, Blood Substitutes Program, Baxter Healthcare Corporation, Round Lake, Illinois, USA 60073. J. A. Boose Ph.D., R. Horner, B. Lu M.D., L. Pearson, Process Validation Division, Microbiological Associates, Inc., Rockville, Maryland, USA 20850.

The heat treatment step used in the production of Diaspirin Crosslinked Hemoglobin (DCLHb) was validated for virus inactivation in a series of experiments representative of the production process scaled down by a factor of 1:680 while duplicating essential solution characteristics such as total hemoglobin concentration, methemoglobin content and oxyhemoglobin level prior to heat treatment, and pH (at 37°C), total hemoglobin concentration, methemoglobin content, degree of crosslinking and yield after heat treatment.

DCLHb reaction mixture (pH adjusted, crosslinked hemoglobin solution prior to heat treatment) was inoculated with a relevant virus at 37° C, heated to 74 ± 1° C over a 30-minute period, incubated at 74 ± 1° C for 90 minutes, and cooled to less than 10° C over a 30-minute period. The amount of viable virus in the solution was determined periodically throughout the heat treatment procedure. Five different viruses (Bovine Viral Diarrhea Virus, BVDV; Pseudorabies Virus, PRV; Human Immunodeficiency Virus 1, HIV-1; Porcine Parvovirus, PPV; and Hepatitis A Virus, HAV) were tested, in duplicate, using process solutions from 6 different manufacturing lots.

The essential solution characteristics throughout the timecourse of the scaled system were consistent with the values determined at the manufacturing scale in each case, except methemoglobin, which was increased by shipping and handling, indicating the similarity of the scaled down test system with the full-scale manufacturing process.

Evaluation of the test solutions throughout the heat treatment process showed greater than 7.2 log reduction for each virus: PRV > 8.8, PPV 7.9, BVDV >8.4, HIV > 7.9 and HAV > 7.2. This exceeds the level of virus inactivation during a single step recommended in the European Committee for Proprietary Medicinal Products guidelines.

To determine if crosslinking affected virus inactivation, process solutions obtained prior to crosslinking were inoculated with either PPV (non-enveloped virus) or BVDV (enveloped virus) and crosslinking agent was added to the test solution. The test solutions were heat-treated and tested as described above. The degree of crosslinking of the final product was 100% and yield of hemoglobin recovery was normal. The essential solution characteristics of the test samples duplicated those obtained from the full-scale manufacturing process. The virus reduction factor was > 6.7 for BVDV and > 8.7 for PPV, similar to those observed in the previous studies. Neither the stability of the virus nor the degree of crosslinking was altered by the presence of virus during the crosslinking process when subsequent heat treatment was performed.

THE VIEW OF JEHOVAH'S WITNESSES ON BLOOD SUBSTITUTES

Richard Bailey, Tomonori Ariga
Hospital Information Services, Watchtower Society, Kanagawa Pref., Japan

Jehovah's Witnesses are very much interested in, and appreciate the efforts being made to develop non-blood derived blood substitutes. This is because Jehovah's Witnesses refuse blood transfusions for religious reasons. They worship Jehovah God and want to live in harmony with his word, the Bible. The Bible tells us to "keep abstaining from . . . blood." (Acts 15:28, 29) However, they are not anti-medicine nor do they desire to exercise the 'right to die.' Rather, they want to live and seek quality alternatives to blood. Therefore, they are very much interested in the development of blood substitutes.

Based on their religious understanding, they do not accept whole blood, and major components of blood, namely, red blood cells, white blood cells, platelets and plasma. However, many Witnesses accept minor derivatives of blood such as albumin, immune globulins or clotting factors. They do not accept the use of blood substitutes that contain hemoglobin, that is a major part of red blood cells. However, each individual Witnesses can decide whether to accept or not a blood substitute that does not contain a major component of blood.

When a new drug is developed, clinical trial is performed. It is necessary to get the patient's informed consent when using such drugs. Needless to say, the same principle applies to the use of blood or its substitutes. Recently, the Welfare Ministry of Japan decided to require the doctors to get the patient's written informed consent for blood transfusions. However, many doctors are still inclined to give blood to Jehovah's Witnesses in emergencies regardless of their explicit refusal, for example, trauma cases or unexpected massive bleeding during surgery. This is not ethical, nor is it legally acceptable. If blood substitutes that Jehovah's Witnesses can use are developed, that would be good news for both doctors and Jehovah's Witnesses. Also, considering the numerous hazards and complications in using blood, that would be of benefit to all.

SAFETY AND EFFICACY OF HEMOGLOBIN MODIFIED BY CROSS-LINKING OR POLYMERIZATION

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Modification of hemoglobin (Hb) is required for the efficacy and safety as blood substitute. Intramolecular cross-linking and polymerization are the two modifications that have been investigated most extensively. In our laboratory, we have first evaluated the intramolecular cross-link of the ß-chains. More recently, we developed a modification by polymerization with glutaraldehyde in order to achieve a further prolonged retention time. Under proper conditions, both intramolecular cross-linking and polymerization is obtained. The half life of this product (PolyHbXl) in man is expected to be more than 24 h. In small animal models we observed that damage to the kidneys and activation of the coagulation system could be prevented by proper modification and purification of the Hb solutions under GMP (good manufacturing practice) conditions. However, we observed consistently a rise in blood pressure in these models. This side effect has to be taken into consideration. From these data we concluded that polymerized Hb products like CLB PolyHbXl potentially meet the requirements of a red cell substitute to be used as a resuscitative fluid under military or civil emergency conditions. Safety and efficacy was further evaluated in a preclinical study including larger animals: pigs and monkeys. After treatment of hypovolemic shock in the pigs, PolyHbXl gave perfusion of tissues and recovery of hemodynamic parameters similar to autologous blood with the exception of a small increase in the pressure in the pulmonary system. The monkey model was a stressed model characterized by a period of hypotension and induction of an acute phase reaction. No serious side reactions were observed in 4 out of 5 monkeys. However, 1 of the 5 monkeys showed a hemorrhagic side effect. Further analysis of the data learned that a related phenomenon was observed in 2 out of 18 rats. The effect was analyzed in a histopathological study with rats. Hemorrhagic lesions were observed in several tissues especially in the intestinal wall two days after administration of PolyHbXl. Microscopically, the bleedings were characterized as "small vessel vasculitis" with a neutrophil infiltration. Endothelial cell injury probably plays a role in the pathophysiology. We found evidence that the toxic factor is a result of the cross-linking with glutaraldehyde. Specially adapted preparation conditions could prevent the effect. Furthermore, Hemolink, a product similar to PolyHbXl but prepared by cross-linking with o-Raffinnose, did not cause this side effect. On basis of these preclinical data it can be concluded that polymerized Hb solutions have a potency as a blood substitute. Some side effects, like the increase in blood pressure, have to be evaluated carefully in order to assess the risk in the use for specific applications.

YEAST-DERIVED RECOMBINANT HUMAN ALBUMIN (RECOMBUMIN™)

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A number of therapeutic proteins that were formerly only available as plasma-derived products are now becoming available as recombinant products. This trend will be discussed and will be exemplified with one of the greatest challenges in this field: the production of high purity recombinant human albumin by an economically viable process.

Recombinant human albumin (Recombumin™ rHA) is produced in the yeast *Saccharomyces cerevisiae*, and purified by a series of chromatographic steps to yield a very pure, consistent product. The molecule has been extensively characterised to verify that rHA is structurally identical to serum-derived albumin (HSA). Detailed analysis has shown that rHA is significantly more homogeneous than HSA.

The Recombumin™ rHA product provides an opportunity to replace HSA not just in therapeutic indications, but also where it is used in the manufacture of other therapeutic products, for instance as an excipient in the formulation of other recombinant products.

A STABLE LONG-TERM MODEL WITH SPONTANOUSLY BREATHING GUINEA PIGS TO EVALUATE ARTIFICIAL OXYGEN CARRIERS FOR MAN UNDER DROPERIDOL-FENTANYL-URETHAN ANAESTHESIA ASSESSING CARDIOVASCULAR, RESPIRATORY AND BLOOD PARAMETER

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Usually artificial oxygen carriers for man are evaluated in rats, because this is a well established and stable small animal model, e.g. (1). But for this purpose the rat owns a very unfavourable half saturation pressure (P50), which is about 35 Torr. This value is 26 Torr in man. In contrast, the P50 value of the guinea pig is similar to that of human. The P50 value of the recipient relative to that of the carrier is a decisive parameter for oxygen release to tissues. On the other hand, it is a known problem to get a reliable anaesthesia in guinea pigs without depression of physiological functions: commonly used narcotics have low therapeutic ranges and non-stability in physiological parameters is likely to appear due to unsuitable drug dosage. A neuroleptic analgesia with a small dose of pentobarbital sodium is recommended (2). We have used urethan instead of pentobarbital sodium because higher stability in physiological parameters had been observed (unpublished data). The following combination of drugs for anaesthesia is used: 10 mg/kg bw (body weight) **droperidol**, 0.2 mg/kg bw **fentanyl** and 400 mg/kg bw **urethan**. Drugs are mixed in isotonic solution and given intraperitoneally.

In 5 guinea pigs (850 to 1050 g bw) the trachea was cannulated and connected to a special micro valve system (3) which allows to determine the respiration frequency, the ventilation and the concentration of expiration gases. Arteria femoralis and right ventricle were catheterised to measure arterial pressure and to obtain arterial and venous blood samples. The preparation takes approximately one hour, after another hour waiting-period measurements began under relatively light anaesthesia. Anaesthesia could be maintained using 20-30 % of the initial dose and 4-5 ml ringer solution per hour. Thus the physiological parameters remain unchanged for additional 6-7 hours. The following values are found: **ventilation** 212.0 $\pm$5.5 ml/min/kg bw; **carbon dioxide release** 7.2 $\pm$0.2 ml CO_2 (STPD) /min/kg bw; **oxygen uptake** 8.1 $\pm$0.3 ml O_2 (STPD)/min/kg bw; **respiratory quotient** 89 $\pm$1 %; **respiration rate** 54.4 $\pm$3.2 /min; **arterial oxygen content** 20.6 $\pm$0.4 ml O_2 (STPD)/dl; **venous oxygen content** 15.2 $\pm$0,9 ml O_2 (STPD) /dl; the **mean arterial blood pressure** was 53 $\pm$5 Torr which is also reported as the normal value (53.1 $\pm$4.2 Torr) in awake guinea pigs (4). **Heart rate** (276 $\pm$6 min) slightly decreased after 4-6 hours to 258 $\pm$6 /min. **Blood-Hb content** was initially 15.6 $\pm$0.3 g/dl. This value also decreased (0.8 $\pm$0.2 g/dl) slightly depending on the number of blood samples taken for measurements.

In conclusion, it seems possible to evaluate in this guinea pig model artificial oxygen carriers for man and it may be also suitable for other studies.

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HEPATIC INFLAMMATORY RESPONSES TO αα-CROSSLINKED HEMOGLOBIN INFUSION IN RATS.

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αα-Crosslinked hemoglobin (αα-Hb) may be a useful alternative to normal blood if it can be safely administered. However, hemoglobin, outside of the red blood cell, has inherent oxidative properties which may lead to oxidant mediated toxicity. Since the liver is the main tissue involved in clearance and metabolism of hemoglobin, this organ was selected for evaluation of whether αα-Hb causes oxidative stress and/or stimulates the immune system to release potentially damaging endogenous mediators such as nitric oxide and cytokines. Previous work in this laboratory has shown that αα-Hb doses up to 1.0 g/kg did not cause liver toxicity. There was also no evidence of oxidative stress, as measured using whole liver samples. In the present study, a higher dose of αα-Hb (2 g/kg or 30 mL/kg, i.v.) was used and indices of inflammation and oxidative stress in individual liver cell types were measured. This higher dose of αα-Hb did not cause liver toxicity; there were no significant increases in plasma alanine aminotransferase (ALT) activity in rats 3 h (50.8 ± 11.0 U/L) or 24 h (33.2 ± 4.6 U/L) after infusion compared to control rats (32.9 ± 3.9 U/L). Possible inflammation after αα-Hb treatment was examined by measuring induction of inflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and cytokine-inducible proteins, nitric oxide synthase 2 (NOS2) and heme oxygenase-1 (HO-1). Induction of these inflammatory proteins was assessed by comparing expression of the gene or protein in liver cells isolated from control or αα-Hb treated rats via reverse transcriptase-PCR or western blotting, respectively. Infusion of αα-Hb increased the expression of both HO-1 gene and protein above control levels in all liver cell types examined. Although Kupffer cells from control rats expressed IL-1β, TNF-α, and NOS2 genes, these compounds were not induced further after infusion of αα-Hb. Liver endothelial cells isolated from rats 3 h or 24 h after infusion of αα-Hb expressed small increases in all cytokines and NOS2 compared to endothelial cells isolated from control rats. Kupffer and endothelial cells isolated from rats treated with LPS (10 mg/kg, i.v.) or *C. parvum* (28 mg/kg, i.v.) and LPS (15 μg/kg, i.v.), to elicit well-established inflammatory reactions in the liver, demonstrated significant induction of genes or proteins for all compounds and were used as positive controls in this study. The absence of both liver toxicity and Kupffer cell stimulation after αα-Hb indicate that αα-Hb does not initiate a strong inflammatory response in the liver. Therefore, induction of HO-1 in all cell types after αα-Hb, is most likely caused by αα-Hb-derived heme and Fe^{3+}, potent inducers of HO-1, rather than by inflammatory cytokines. The small induction of inflammatory cytokines and NOS2 in endothelial cells upon αα-Hb administration suggests that the endothelium may be uniquely susceptible to the damaging effects of high-dose hemoglobin. Whether this response is mediated by hemoglobin-derived oxidants remains to be determined, but suggests that pre-existing inflammation may predipose individuals to αα-Hb induced oxidative damage. (Supported by NIH/NHLBI grant #53030-02)

ZERO-LINK POLYMERIZATION: A NEW CLASS OF POLYMERIC HEMOGLOBINS.

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Human and bovine hemoglobins can be polymerized by intermolecular crosslinking based on the activation of the carboxyl groups on the surface of the tetramers with 1-ethyl-3-(3-dimethyl- aminopropyl) carbodiimide. The active carboxyl groups form pseudopeptide bonds with the amino groups present on the surface of an adjacent molecule. Extensive polymerization can be obtained with both human and bovine hemoglobins. Before this treatment human hemoglobin is intramolecularly crosslinked with a sebacoyl residue between the β82 lysines. In analogous fashion bovine hemoglobin is previously crosslinked with an adipoyl residue. The oxygen affinity of the resulting polymers is proportional to the extent of polymerization. Polymers can be obtained with P50 near 20 mmHg for human hemoglobin and near 30 mmHg for bovine hemoglobin. The oncotic activity of these compounds is strongly dependent on protein concentration. The various preparations are isooncotic at 8-9 g/dL. Their molecular weight ranges from 120,00 to 1,000,000. After a 30% exchange transfusion in the rat they are not eliminated with the urine and have a half time of intravascular retention of about 18 h. As usual with hemoglobin derivatives the blood pressure shows a 10-20% transient increase after infusion. No indication is noticed of kidneys or heart discomfort.

DIASPIRIN CROSSLINKED HEMOGLOBIN: EFFICACY AND SAFETY FOR CEREBRAL VASOSPASM

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Introduction: We evaluated the effect of α-α diaspirin crosslinked hemoglobin (DCLHb™) on cerebral ischemia and brain injury after subarachnoid hemorrhage (SAH), and the effect of subarachnoid administration of DCLHb on cerebral blood flow (CBF) in rats after SAH.
Methods: SAH was induced by injecting 0.3 ml of autologous blood into the cisterna magna.
Part A: The rats were assigned to one of the following groups:

Control (n=24)-no hematocrit (45%) manipulation;
DCLHb (n=24)-hematocrit decreased to 30% with 10% DCLHb;
Alb (n=24)-hematocrit decreased to 30% with human serum albumin.

Eight animals in each group received a single treatment, while for 8 animals treatment was sustained for 48-hr. 48-hr after SAH the area of hypoperfusion was assessed with ^{14}C-iodoantipyrine. For 8 animals in each group the number of dead neurons was determined.
Part B: After SAH the animals received one of the following solutions into the cisterna magna:

Control (n=8)-0.3 ml of mock cerebral spinal fluid.
Blood (n=8)-0.3 ml of fresh autologous blood.
DCLHb (n=8)-0.3 ml of 10% DCLHb.
Alb (n=8)-0.3 ml of human serum albumin.

20-min after subarachnoid injection of one of the above solutions, the area of hypoperfusion was determined. The data were evaluated by ANOVA, and mean values compared by t-tests.
Results: Part A: the area of hypoperfusion was less in the DCLHb and Alb groups vs the Control group. The number of dead neurons was less in the Alb group vs the Control group, and was less in the DCLHb group vs the other two groups (see Table). Part B: The area of hypoperfusion was greater in the Blood group vs the Control, DCLHb, and Alb groups. The area of hypoperfusion was greater in DCLHb group vs the Control group (see Table).
Discussion: These data support the hypothesis that hemodilution decreases hypoperfusion after subarachnoid hemorrhage, and that in terms of neuronal death, DCLHb is a more effective hemodiluent than albumin. Moreover, the present data support the concept that although extravasated molecular hemoglobin decreases CBF, the potential adverse effect is not as great as whole blood. The present data do not support the notion that intravascular molecular hemoglobin has an adverse effect on ischemic brain injury after subarachnoid hemorrhage.

	Control	DCLHb	Alb	Blood
Part A				
Hypoperfusion Area (single treatment)	19±4	9±3*	8±2*	---
Hypoperfusion Area (sustained treatment)	18±4	2±1*	3±1*	---
Dead Neurons	1097±211	305±38†	611±84*	---
Part B				
Hypoperfusion Area	26±5	34±6*	30±6	75±9‡

Table-area of hypoperfusion (CBF<40 ml·100g^{-1}·min^{-1}) in a coronal brain section (% of total area, mean±SD) for Parts A and B; and the number of dead neurons for each group. *p<0.05 vs the Control group. †p<0.05 vs the other two groups. ‡p<0.05 vs the other three groups.

OVERVIEW OF THE EFFECTS OF DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb) ON OXYGENATION AND PERFUSION OF THE MICROCIRCULATION.

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Background: The perioperative period following a high blood loss surgery and the immediate period following traumatic hemorrhagic shock are believed to share the common sequela of inadequate tissue perfusion and tissue dysoxia. Currently, there are a number of products that function primarily as temporary volume expanders, and thus, attempt to transiently increase blood flow to tissues (e.g., a number of different crystalloids and colloids). However, there is still a great deal of morbidity and mortality observed in both of these clinical settings. Diaspirin crosslinked hemoglobin, a hemoglobin based oxygen therapeutic, not only possesses many of the same properties as these other resuscitation solutions, but in addition, has novel pharmacologic properties and excellent oxygen transport properties. Therefore, the potential efficacy of DCLHb was examined in a number of animal models designed to assess tissue perfusion as well as oxygenation.

Methods: To investigate perfusion, a variety of different experiments were conducted in rats, hamsters, and swine subjected to either hemorrhagic shock or made critically ill by induction of sepsis. Prior to resuscitation/treatment, and at a variety of time periods following resuscitation with either DCLHb or other commonly used resuscitation fluids, perfusion and oxygenation were measured. A number of different techniques were used to assess perfusion:

- Hemodynamic measurements
- Global indices of acid-base status, such as base excess and lactate concentrations
- Radioactive microsphere technique for blood flow measurement
- Measurement of blood flow with ultrasonic flow probes
- Intravital microscopy

A number of other techniques were used to assess tissue oxygenation, including:

- A special palladium porphyrin phosphorescence technique
- Micro-platinum electrodes placed in muscle tissue
- Oxygen sensing electrodes (optodes)
- Measurement of whole body oxygen consumption

Results/Conclusions: The results of a variety of preclinical studies suggest that diaspirin crosslinked hemoglobin, a hemoglobin based oxygen therapeutic, enhances tissue perfusion and oxygenation. In fact, DCLHb is currently being evaluated in pivotal clinical trials in trauma and surgery. An overview of the results of a number of different preclinical studies in this area of research will be reviewed.

RECENT PROGRESS IN THE DEVELOPMENT OF OPTRO® (RECOMBINANT HUMAN HEMOGLOBIN, rHb1.1)

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Recent progress in the development of Optro® (recombinant human hemoglobin, rHb1.1) will be presented. This will include an update on several clinical studies, as well as progress being made with second generation recombinant hemoglobin constructs. An early Phase II clinical trial demonstrated that Optro® could be safely dosed up to 100 gm in an elective surgery indication. In addition, another early Phase II study showed that acute normovolemic hemodilution (ANH) could be safely performed with Optro®.

The current clinical program with rHb1.1 is focused on enhancing ANH in a cardiopulmonary bypass setting, as well as exploring the hematopoietic effects of low dose, repeat administration of rHb1.1 in patients with chronic anemia.

CROSS-LINKED AND ENCAPSULATED HEMOGLOBIN AS BLOOD SUBSTITUTES

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Hemoglobin (Hb) cannot be used as blood substitutes. They break down in the body and they also cause toxicity. To prevent these problems, we have studied the crosslinking Hb(Chang 1964) and microencapsulation of Hb (Chang 1957). HIV has stimulated extensive academic and industrial development of cross-linked hemoglobin since 1986. Cross-linked hemoglobin now consists of intermolecularly cross-linked hemoglobin (Polyhemoglobin); intramolecularly cross-linked hemolgobin; conjugated hemoglobin. Hemoglobin can also be intramolecularly modified using reombinant technology. Unlike rbc, they can be sterilized by pasteurisation, ultrafiltration and chemical means. This removes infective microorganisms responsible for AIDS, hepatitis etc. Since they are free of rbc blood group antigens, there is no need for cross-matching or typing. This saves time and facilities and allows on the spot transfusion like the infusion of plasma expanders.Furthermore, they can be lyophilized and stored for a long time as a stable dried powder. In addition to using Hb extracted from human rbc, other sources of Hb are available. These include bovine Hb, transgenic human Hb and recombinant human Hb. Crosslinked ultrapure Hb and recombinant human Hb are less complicated than encapsulated hemoglobin, thus several groups are now in Phase II to Phase III clinical trials. Potential applications include their use in cardiac, cancer, orthopedic and other surgery, traumatic injuries with severe hemorrhage and other areas. Success in these first generation blood substitutes has stimulated the development of second generation blood substitutes. For example, a number of approaches are being explored to reduce oxygen radicals in reperfusion injuries. Our group is studying the crosslinking of Hb to superoxide dismutase and catalase to reduce oxygen radicals especially in reperfusion injury. Encapsulated hemoglobin is an even further generation of hemoglobin based blood substitutes. This is a more complete artificial red blood cell (ARBC). For example, like rbc, hemoglobin inside ARBC is not exposed to the external environment. ARBC can enclose hemgolobin with all the enzyme systems including superoxide dismutase, catalase, carbonic anhydrase, methemoglobin reductase and the various metabolic cyclic enzyme systems. Furthermore, by modifications of the surface properties of ARBC, the circulation time can be increase to much higher than that of cross-linked hemoglobin. Those formed with lipid membrane vesicles are well into the final stages of animal studies. Success in hemoglobin lipid vesicles have stimulated the search for even more futuristic type of artificial red blood cells. For example, we have been studying the use of biodegradable polymer (e.g. polylactic acid) and nanotechnology to form artificial red blood cells of nanodimensions with ultrathin biodegradable membranes. By variations in the polymer composition and molecular weight, biodegradable polymer can biodegrade at selected rates in the body. In the case of polylactic acid, it is degraded into carbon dioxide and water without any loading of the RES.

CLINICAL EXPERIENCE WITH PYRIDOXALATED HEMOGLOBIN POLYOXYETHYLENE (PHP)

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Pyridoxalated Hemoglobin Polyoxyethylene (PHP) is being developed as a nitric oxide scavenger for use in nitric oxide (NO)-induced shock. NO-induced shock occurs at high frequency in critically ill patients as a result of the inflammatory response to infection and other insults. PHP has been tested in healthy volunteers at doses up to 100 mg/kg and was shown to be safe and well tolerated. Similar studies in septic shock patients have demonstrated significant increases in mean arterial pressure at doses of 50 and 100 mg/kg, as well as decreases in concurrent vasopressor utilization. Current trials in septic shock patients are in progress, investigating the effect of continuous infusion.

A SYSTEM ESTABLISHING COMPATIBILITY PROFILES FOR ARTIFICIAL OXYGEN CARRIERS AND OTHER SUBSTANCES

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Many artificial substitutes for blood or blood components have been developed so far, some of which are designed to carry oxygen. Toxicological and immunological compatibility is generally tested largely using animal experiments. In particular inflammatory parameters however show large species specific differences. We therefore developed an in vitro system using mainly human components to analyze the inflammatory potential of a given substance. It can be used for quality control purposes with known compounds and for the assessment of the inflammatory/compatibility profile of unknown substances.
The test system comprises induction of hemolysis, activation of complement (C3a), induction/suppression of cytokine production, direct toxicity for different cell lines and human leukocytes and finally phagocytosis of the material under test and effects on phagocytosis of microbes. Phagocytosis and effects on vitality and function of phagocytes were measured using a fluorescense activated cell sorter. When testing lecithin-based perfluorodecalin (PFD) emulsions in comparison to a pluronic-based PFD-emulsion we could show, that pluronic-based emulsions where basically untoxic with different cell lines, including peripheral human leucoytes, caused mild inhibition of endotoxin-induced cytokine production, but were strongly phagocytosed by granulocytes and macrophages. At the same time lecithin-based PFD-emulsions were phagocytosed with considerably lower rate. They did however cause moderate cytotoxicity in phagocytic cells like monocytes (>60% after 24h incubation, 100% after 48h) and granulocytes (>15% after 24h incubation, >80% after 48h). Lecithin based emulsions also suppressed endotoxin induced cytokine production in monocytes to more than 80%.
Other newly synthesized compounds (partially fluorinated carbohydrates) which had proven to cause inflammation in animal experiments showed fast and pronounced toxicity for phagocytic cells in our test system, but little or no toxicity with several cell lines of lymphatic or epithelial origin.

OXYGEN DELIVERY BY RECOMBINANT HUMAN HEMOGLOBIN VARIANTS

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Using a series of recombinant human hemoglobin variants having reduced rates of reaction with nitric oxide (NO), we have shown a diminution of the pressor response that is correlated with the rate of NO scavenging. Concomitant with modifications of NO reactivity are alterations of both O_2 kinetics and affinity. Since O_2 delivery to the tissues likely depends on binding kinetics as well as O_2 affinity, this study was carried out to assess the efficacy of O_2 delivery for a series of recombinant hemoglobin variants possessing a range of P_{50} values and O_2 and NO binding constants. Male Sprague-Dawley rats were anesthetized and instrumented with indwelling catheters in the right ventricle, descending aorta, and vena cava for hemoglobin infusion, blood withdrawal, arterial pressure measurement, and maintenance of anesthesia. The animals were sealed in a respirometer for continuous measurement of O_2 consumption. Arterial pressure, heart rate, and body temperature were also monitored continuously throughout the experiment. Periodic arterial and venous blood samples were taken for measurement of blood gases, O_2 content, hemoglobin concentration, and hematocrit. Cardiac output was calculated from O_2 consumption and the arteriovenous O_2 content difference. After approximately 60 minutes for stabilization and recording of baseline values, complete isovolemic exchange transfusion was carried out at a rate of 3 ml/min/kg with hemoglobin variants (5g/dl) or 5% human serum albumin (HSA) until hematocrit fell below a nominal value of 2%. Measurements continued for 20 minutes following cessation of exchange. In all experiments, hematocrit fell monotonically from starting levels of approx. 45% to below 2% within 60 minutes. At the end of exchange with hemoglobin variants, circulating levels of recombinant hemoglobin were approximately 4 - 4.5 g/dl. In the animals that received HSA, O_2 consumption fell dramatically as hematocrit declined below 20%, and none of the animals survived to the end of exchange. As expected, arterial base excess and the arteriovenous O_2 content difference also declined markedly. O_2 extraction rose initially before declining as the hemoglobin concentrations fell to low levels (< 3 g/dl). When hemoglobin variants with higher P_{50} values were used (> 20 mmHg), O_2 consumption remained at near-control levels. Despite slower O_2 dissociation rates, O_2 extraction increased to compensate for the low circulating hemoglobin concentrations and allowed the arteriovenous O_2 content difference to be maintained. As a consequence of low hemoglobin concentrations, venous PO_2 also declined. O_2 consumption was similar in animals that received hemoglobin variants with low P_{50} values (< 10 mmHg). However, O_2 extraction did not increase sufficiently to maintain the basal arteriovenous O_2 content difference. In addition, venous PO_2 declined further than observed with molecules having higher P_{50} values despite similar hemoglobin concentrations. It thus appears that reductions in O_2 dissociation rates can be tolerated if O_2 affinity is kept low. From these results, we conclude that the kinetics of NO scavenging can be significantly altered without severely impacting O_2 delivery under basal metabolic conditions.

COMMERCIAL ANALYSIS OF BLOOD SUBSTITUTE MARKET

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Blood substitutes have been sought for over 80 years by many researchers. Green Cross obtained the first and only PFC FDA approved blood substitute, Fluosol DA-20%, in 1989 for angioplasty.

The largest markets are transfusion, MI, cancer and stroke plus 100 others, and the benefits are: no donors, no testing for disease, no typing and cross matching, no refrigeration, much longer shelf life and oxygen transfer.

The successful blood substitute has been and can be sold for $300-$600/600 ml unit.

The leading contenders are Alliance, Baxter, Northfield and Somatogen,, followed by BioPure, Hemosol, etc. Major dropouts include Abbott, Upjohn, Pharmacia, Lilly, Synthetic Blood, U.S. Army and Navy, Adamantech, Affinity Biotech, Oxygenectics, DNX, Sun Tech, Hemagen, Burns Lab, Quest and others.

Blood substitutes comprise the world's largest pharmaceutical market, estimated at U.S. $30 Billion/year, and will provide major improvements in health care.

Most research has been on reusing hemoglobin through cross-linking, but raw material availability will be the biggest hurdle in large scale production.

The reasons for dropouts are lack of funding, lack of success in development or problems during clinical trials.

PROPERTIES OF POLYETHYLENE GLYCOL-CONJUGATED RED BLOOD CELLS

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Polyethylene glycol (PEG) is a non-toxic, hydrophilic polymer, which has been widely used to modify proteins, enzymes, drugs and liposomes in order to reduce their immunogenicity and to prolong their circulation times when injected *in vivo*. PEG-modified hemoglobin is currently under development as a potential blood substitute. We have recently developed a technique to covalently bond a brush coating of PEG to the membrane of living red blood cells. When bonded to the RBC membrane, PEG forms a steric barrier which excludes immuno-globulins and other large plasma proteins from the RBC surface, but permits the free diffusion of small molecules (e.g., water, ions, sugars, O_2 and CO_2). The PEG-coating procedure does not cause any apparent morphologic, structural or functional damage to the RBC.

By preventing antibodies from approaching the RBC surface, PEG coating effectively 'masks' blood group determinants. We have examined the effect of PEG-coating of RBC on agglutination reactions to a wide range of specific blood group antibodies. Complete inhibition was observed with antibodies to the rhesus antigens D, c, C, e and E, and to clinically significant antigens from other blood groups: Le^b, Jk^a, P_1 and N. Of the antigens studied, only A, B and I were incompletely masked by PEG, however, the antibody titers required to induce agglutination were still substantially reduced, typically from 1:256 to 1:4.

PEG coating also excludes fibrinogen from the RBC surface, and therefore prevents RBC aggregation, which in turn results in a greatly reduced low-shear blood viscosity. The viscosity of PEG-treated RBC suspended at 40% hematocrit in autologous plasma measured at low shear ($0.1\ sec^{-1}$) was 16 mPa.s compared to 77 mPa.s for control (untreated) RBC. This decrease in viscosity was of similar magnitude to that achieved by hemodilution to a 30% hematocrit with 6% dextran 40 in saline. Note, however, that with PEG-coated RBC the viscosity was reduced without compromising the oxygen-carrying capacity. Studies with mixtures of PEG-coated and non-coated control RBC at 40% hematocrit showed that as few as 10% of treated cells reduced low-shear viscosity by about 25%, while 25% and 50% PEG-RBC reduced the viscosity by 50% and 75% respectively.

Our initial evaluations have thus far been limited to *in vitro* studies, but *in vivo* experiments to examine the survival of PEG-coated RBC are now in progress. We are also developing enhanced PEG derivatives with the intention of also masking the A, B and I antigens. We believe that this technique may eventually prove to have an important role in transfusion medicine: Firstly because of the ability to mask many clinically significant blood group antigens, which may be of value for emergency transfusion, for patients with multiple alloantibodies, or possibly to prevent the development of alloimmunization in patients receiving repeated transfusions; secondly because the reduced low-shear viscosity will facilitate blood flow to ischemic tissues under low flow conditions, e.g., during hemorrhagic shock or vaso-occlusive crisis in sickle cell disease.

RED CELL SUBSTITUTES: EVOLUTION OF APPROACHES TO DEMONSTRATING EFFICACY

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The past decade has seen a striking advancement in our understanding of red cell substitutes. Throughout this period, regulatory oversight influenced many aspects of product development. The approach to demonstrating efficacy was an important example. I will review some events of the decade so that we may consider the question, "If we had it to do over again, could we do any better?"

At the start of the decade, it was felt that there was a sufficient knowledge base to there were sufficient data on the mechanism of hemoglobin-induced renal toxicity to convince investigators that the content of $\alpha\beta$-dimers in any hemoglobin product must be strictly limited. An array of additional, unexpected reactions were seen in early clinical studies, reactions that led to several conclusions: hemoglobin is a pharmacologically active material; more basic studies are needed; and it would all be so much easier if communications between investigators were open and unhindered.

Meaningful studies of efficacy could not be approached until the safety issues had been solved or until the community was convinced that they were not serious. The initial approach was based on the meager experience gained with previous products, e.g., Fluosol. That experience led to policies which required that, for example, if the product was to be used as a red cell substitute, it must be compared with red cells, and if it was to be used as an oxygen carrier, it must be shown to support organ function. Endpoints of clinical trials were based on delivery of a clear clinical benefit to the patient - surrogate endpoints could be used only if their relationship to clinical benefit had been demonstrated.

Now, at the end of the decade, it is time to ask whether progress would have been greater had policies regarding demonstration of efficacy been different. I will examine that question with specific examples and present the possible outcomes.

EFFECT OF LIPOSOME-ENCAPSULATED HEMOGLOBIN ON THE FUNCTION OF HUMAN MONOCYTES IN VITRO

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Liposome-encapsulated hemoglobin (LEH) has been developed as a red blood cell substitute, and the efficacy of this material has been demonstrated in total isovolemic exchange transfusion, hemorrhagic shock and hemodilution state in animal models. Because the reticuloendothelial systems as well as lung are largely responsible for removal of LEH from the circulation, the effects of LEH on the immunoresponse of the fixed tissue macrophages have been well studied. Biodistribution study using radio-labeled LEH showed significant retention of LEH (43%) in the blood at 20 h after administration. The observation prompted us to study the effect of LEH on the function of human monocytes. Peripheral blood mononuclear cells (PBMCs) were cultured in RPMI-1640 containing 10% FCS with 5 % (v/v) of either saline or LEH. Electronmicroscopic examination demonstrated the presence of LEH in the monocytes incubated with LEH. Flow cytometric analysis showed that the side-scatter increased as the concentration of LEH increased. These results suggested that monocytes in fact phagocytosed LEH. The viability of monocytes was assessed by double staining with propidium iodide and anti-CD14 antibody. The increase of LEH concentration (1%-5%) resulted in the significant decrease of viability, whereas the viability of CD14 negative cells remained relatively constant. The reduction of viability of monocytes by LEH was further enhanced by the coincubation with monocyte-activator (i.e. interferon-γ or LPS). These results may implicate that LEH administration leads to selective cell reduction of monocytes in the circulation.

COMMERCIAL MANUFACTURING OF BLOOD SUBSTITUTES: FUTURE ISSUES

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As the clinical development of hemoglobin-based products has progressed, the successful commercial manufacture of these products has become an important issue. The major challenge is to develop a cost-effective manufacturing strategy to produce very large quantities of these products. This presentation will review various options for the sourcing of hemoglobin as a raw material and cost issues related to down-stream processing and production of the finished product. Discussion will include both recombinant and human red cell-based blood substitute products.

The large scale production of Neo Red Cell

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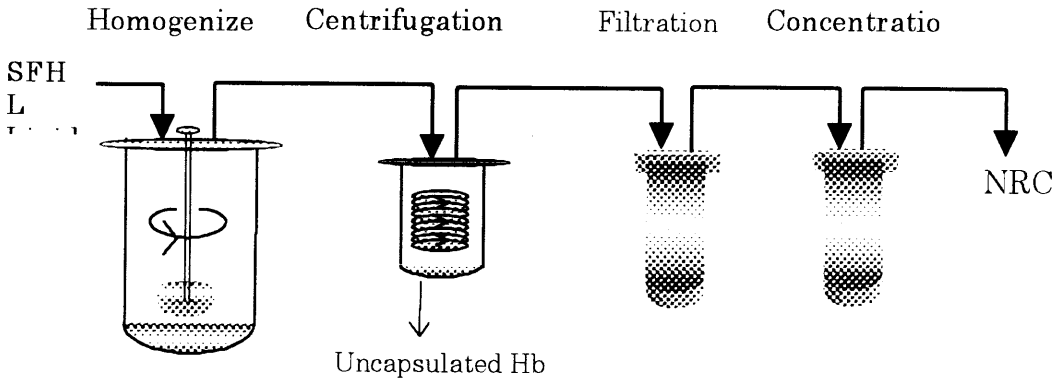
We have developed and evaluated the liposome encapusulated hemoglobin (Neo Red Cell (NRC)) as the blood substitute. The NRC was the liposome encapsulated high-concentrated (>45%) stroma free hemolysate (SFHL) with inositol hexaphosphate (as an allosteric effector), coenzyme and substrates for reducing metHb. And the NRC surface was coated with polyethylene glycol to prevent aggregation in plasma.

The NRC production consists of the producing of SFHL and the process of encapsulation hemoglobin in liposome.



The schematic model of the production of Neo Red Cell

In commercial production of NRC as blood substitute, the essential condition is the bulk production technique and facilities. Although the efficient production technique of liposome is very important, the bulk production of liposome is not so easy generally, because of technical difficulty which are emulsification of the mixture of hemoglobin and lipids and the uniformity control of liposome size. And it is important that the sterilization process is maintained, as the sterile for injection and surrounding conditions are hold for aseptic production. Then the technique of NRC production was established that was composed of simple processes and it was confirmed that the production on large scale was possible. We can produce 40 liter/month and aim at the production of 4000 liter/month in near future.

THE CLINICAL UTILITY OF HUMAN POLYMERIZED HEMOGLOBIN AS A BLOOD SUBSTITUTE FOLLOWING TRAUMA AND EMERGENT SURGERY

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We have previously described our experience with the infusion of up to 6 units (300 grams) of human polymerized hemoglobin (PolyHeme™) in a non-randomized trial in trauma and surgical patients. This report describes the first patient trial to directly compare the use of PolyHeme to allogeneic blood in the treatment of acute blood loss due to trauma and emergent surgery.

Methods: 20 patients (17 male, 3 female) aged 19-74 years were randomly assigned to receive either PolyHeme (experimental) or blood (control) when a clinical decision was made that transfusion was indicated. In the experimental group, PolyHeme was infused up to a maximum of 6 units as needed. The allowable "infusion period" ended when surgical hemostasis was obtained. If more transfusions were required, these patients then received blood. The control group received only blood.

Results: There were no significant safety issues. The infusion dose of PolyHeme (4.6 ± 2.0 units) and blood (4.5 ± 1.9 units) were equivalent. The plasma [Hb] due to PolyHeme at end-infusion was 3.7 ± 1.2 g/dl. The [Hb] data (g/dl) before and after infusion are shown below:

	PRE-INFUSION	
	Total [Hb]	Red Cell [Hb]
Experimental	9.4 ± 2.0	9.1 ± 1.9
Control	10.6 ± 2.0	10.2 ± 1.9

	END-INFUSION	
	Total [Hb]	Red Cell [Hb]
Experimental	8.5 ± 1.7	5.2 ± 1.9
Control	10.3 ± 1.0	10.0 ± 1.1

PolyHeme maintained total [Hb], despite the marked fall in red cell [Hb]. The experimental group received less allogeneic blood (8.4 ± 3.4 units) than the controls (13.3 ± 4.5 units) during their hospital stay.

Conclusion: PolyHeme effectively maintains total [Hb] in lieu of red cells following acute blood loss, and thereby reduces allogeneic transfusions. PolyHeme appears to be a clinically useful blood substitute.

RED CELL SUBSTITUTES IN PERSPECTIVE: LEARNING FROM THE PAST AND APPLYING IT TO THE FUTURE

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The history of red cell substitutes is nearly one hundred years old. There have been distinct and obvious phases marked by scientific and technologic advances yet the fundamental issue of delivery of oxygen to tissues remains paramount as the driver of development. Chemical modification of hemoglobin is one means of attaining the desired goal. This presentation will review the basis for chemical modification and place a perspective on lessons learned and future developments.

BLOOD PROGRAMS IN ASIA AND OCEANIA: KOREA

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The number of whole blood donation has increased gradually and we are no more short of red cells and platelet concentrates in Korea. The Korean Red Cross Blood Centers collect more than 95% of blood. About 85% of blood are drawn from young male donors, mainly from army forces and high schools. We are importing about 300,000 liters of plasma a year for the production of plasma fractionation products such as albumin and factor VIII. The Headquarter of Blood Program in the Korean Red Cross recently recruited 11 young doctors to support the program and has set several imminent goals like below:

1. Increase the number of apheresis donations for platelets and plasma
2. Recruit more number of individualized regular donors
3. Construct computer network system between major donor centers and transfusion services
4. Standardize component preparation and donor screening technology
5. Set a national guideline for blood donation and transfusion practices
6. Expand donor pool for for the Korean Bone Marrow Registry
7. Support research activities related to transfusion medicine

EXPERIMENTAL AND MATHEMATICAL SIMULATION OF OXYGEN TRANSPORT BY HEMOGLOBIN-BASED BLOOD SUBSTITUTES

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Introduction

Solutions of extracellular hemoglobin and especially, polymers of hemoglobin have received a great deal of attention as potential blood substitutes. Because of the complexities of *in vivo* studies, an experimental system for *in vitro* simulation of oxygen transport to tissue has been created.

Methods

The system includes a 25 μm diameter "capillary" embedded in a thin silicone rubber film. The capillary is cannulated at each end and perfused with red blood cell suspensions, hemoglobin solutions, polyhemoglobin solutions or mixtures thereof. The capillary is mounted on the stage of a microscope, and a dual wavelength microspectrophotometric technique is used to determine the oxygen saturation of the sample at various axial positions. The oxygen tension of the sample and of the extracapillary space is controlled to simulate oxygen uptake in the lung or oxygen release to the tissue.

Results

Results are obtained as oxygen saturations of the specimens at various axial positions for a wide range of flow rates of sample through the capillary. The results for the various flow rates can be all brought together when expressed as a function of residence time in the capillary. Solutions of hemoglobin or of hemoglobin polymer transport oxygen much more rapidly than red blood cell suspensions with the same hemoglobin content. Replacement of red cells with relatively small amounts of extracellular hemoglobin or hemoglobin polymer significantly enhances oxygen transport. For example, in some experiments 10 percent of the red cell hemoglobin was replaced with an extracellular blood substitute while maintaining the same total hemoglobin content. Under some circumstances this replacement yielded a 50 percent increase in oxygen release.

The experiments are simulated with a predictive mathematical model which is shown to be in good agreement with the experimental results.

Conclusions

The *in vitro* and theoretical simulations are useful methods for evaluation of the oxygen transport characteristics of blood substitutes. The results show in a quantitative way that relatively small amounts of hemoglobin-based blood substitutes yield substantial enhancement of oxygen transport.

POLYNITROXYL-HEMOGLOBIN (PNH) AS AN OXYGEN CARRIER AND REPERFUSION THERAPEUTIC

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The nitroxide moieties of polynitroxyl-hemoglobin (PNH) can scavenge toxic free radicals by mimicking the activities of enzymes including superoxide dismutase, catalase, ferryl reductase. PNH can deliver oxygen and is free of the vasconstrictive activity associated with other hemoglobin-based oxygen carriers. PNH has commercial potential both as a blood transfusion fluid and as a reperfusion therapeutic in clinical indications relating to ischemia/reperfusion injury. Such indications include stroke, myocardial infarction, and perioperative ischemia. Clinical trials of PNH are projected to begin in 1998, and preclinical safety and efficacy data will be discussed.

PLATELET-LIKE PARTICLE PRODUCTION FROM CULTURED HUMAN MEGAKARYOCYTIC CELL LINE

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Artificial platelet substitutes have been developed in several manners; lyophilized platelet vesicle, purification of platelet membrane, platelet glycoprotein-coated erythrocyte (thromboerythrocyte), platelet glycoprotein-coated liposome (plateletsome) and so on. As another direction of the development, we investigated the biological characteristics of human megakaryocytic cell line. CMK cell line derived from a Down's syndrome patient showed several characteristics similar to bone marrow megakaryocytes, including the expression of platelet-specific glycoproteins, the development of demarcation membrane system and the production of platelet-like particles. The presence of a few alpha-granules was also reported. Initially CMK cells expressed GPIIb/IIIa more than 80%, whereas the expression of GPIb was less than detection level. CMK cells cultured in the presence of interleukin-3 (IL-3) and anti-viral drug Ribavirin began to express GPIb at a culture period-dependent manner without a cease of proliferation. Ribavirin, when administered in vivo, increased the platelet number in the peripheral blood in some clinical trials. IL-3 and thrombopoietin (TPO) which possess both proliferation and differentiation capability toward megakaryocytes did not enhance GPIb expression. Moreover, a large number of platelet-like particles were produced as well. CMK cells stained by membrane dye PKH26 produced dye-positive particles, which indicated that the particles were consisted of the cellular membrane. Separated particles had a capacity to adhere to the collagen coated plate in the presence of human plasma. While several important investigations should be cleared including the aggregation abilities in vitro and the hemostatic capability in vivo, recent advances in the large-scale cell culture systems may enable this megakaryocytic cell line to supply a large number of platelet-like particles.

LIPOSOMES CARRYING VON WILLEBRAND FACTOR-BINDING DOMAIN OF PLATELET GLYCOPROTEIN Ib α ENHANCE RISTOCETIN-INDUCED PLATELET AGGREGATION.

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Platelet glycoprotein (GP) Ib/IX complex is a receptor for von Willebrand factor (vWF), which plays a crucial role in primary hemostasis. We have previously expressed in CHO cell a domain of GPIb α (residues 1-302 of mature GPIb α) that retained a vWF-binding function. Based upon a hypothesis that liposomes capable of participating in the hemostatic process may become possible platelet substitutes, we have attempted to construct liposomes carrying von Willebrand factor-binding domain of platelet glycoprotein Ib α. We have incorporated this recombinant fragment (rGPIb α) into liposomes composed of egg lecithin, cholesterol, and phosphatidylgylycerol (10:5:2, by molar ratio) and evaluated their functons in *in vitvo*. rGPIb α on the liposome surface was detectable by flow cytometry using an FITC-labeled anti-GPIb α monoclonal antibody, GUR20-5. Agglutination of rGPIb α-liposomes was monitored by an aggregometer PA-100 (Kowa, Japan), that measures changes in light scattering. Addition of vWF and ristocetin caused specific agglutination of rGPIb α-liposomes, which was completely abolished by an anti-vWF monoclonal antibody NMC-4 (a generous gift of Dr. A Yoshioka). Fluorescent microscopy showed that rGPIb α-liposomes labeled with rhodamine agglutinated in the presence of vWF and ristocetin. We next examined whether heterologous aggregation, i.e., attachment of liposomes to platelets, would occur. Platelet-rich plasma (PRP) was first mixed with rhodamine-labeled rGPIb α-liposomes, and ristocetin was added to induce platelet aggregation. Rhodamine-fluorescence was strongly positive in platelet aggregates, but was negative when rhodamine-labeled control liposomes were used. When rGPIb α-liposomes were mixed with PRP at low platelet concentration (20-80 $x10^3/\mu$ l), rGPIb α-liposomes dose-dependently enhanced ristocetin-induced platelet aggregation as assessed by PA-100. In summary, rGPIb α-liposomes were incorporated into platelet aggregates and enhanced platelet aggregation. rGPIb α-liposomes may bind vWF and accumulate on exposed subendothelial tissue in vivo, serving as platelet substitutes supporting hemostasis in thrombocytopenic individuals.

PARTIAL EXCHANGE TRANSFUSION OF POLYOXYETHYLENE-CONJUGATED HUMAN RECOMBINANT HEMOGLOBIN INTO RAT

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To create a stroma-free oxygen-carrying material suitable for blood substitute we synthesized a human recombinant hemoglobin, EHb V67βI, in which Val-67(E11) of the β chain is replaced by Ile, by protein engineering (Nagai et al., 1985) and attached polyoxyethylene to it to enhance *in vivo* stability (Iwashita, 1991). This chemically modified recombinant Hb, POE-EHb V67βI, showed an oxygen affinity comparable to that of human whole blood. In the present study we examined its *in vivo* behavior, safety and life time by partial exchange transfusion into rat.

From a vein of an SD rat (170 weight) anesthesized with sodium pentobarbital (40 mg/kg), 1 ml of blood was collected and a POE-EHb V67βI solution of the same volume (containing 20 mg Hb) was injected. After 1 min later, 0.5 ml of blood was further collected in exchange for 0.5 ml of physiological saline. Thereafter, this blood sampling was repeated at certain time intervals until about 3 hours elapsed after the first transfusion. Blood pressure, heart rate, temperature at the rectum and oxygenation status in the cerebral tissues were continuously monitored. The oxygenation status was detected as changes in deoxy, oxy and total Hb concentrations and changes in oxidized form of cytochrome oxidase (ox. cyt. aa3) by means of 4-wavelength near infrared spectro-phtometry. The residual Hb in the circulating blood was determined by absorbance measurement of supernatant of collected blood samples after centrifuge. A control experiment was performed using a POE-free EHb V67βI solution.

In both the cases for POE-EHb V67βI and EHb V67βI, the blood pressure, heart rate and oxyHb concentration showed transient decreases during the first 1 hour which was followed by restoration to the original values. In both the cases, the concentrations of deoxyHb, total Hb and ox. cyt. aa3 decreased gradually with time due to stepwise dilution of the circulating blood. The half time of disapearance of Hb from the circulation was 84 min for POE-EHb V67βI and 32 min for EHb V67βI. There was no sign of pyrogenic rise in body temperature. The present study proved that the life time of our recombinant Hb is elongated by 2.6 times by conjugation with POE and POE-EHb V67βI has no significant toxity as long as observed for a short period.

BIOPHYSICAL CRITERIA FOR MICROCIRCULATORY EFFICACY OF BLOOD SUBSTITUTES

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Tissue oxygenation by microvascular blood is an autoregulated phenomenon which insures that under normal conditions the delivery of oxygen matches the rate of consumption throughout the cellular structures. This balance results from the activity of systemic and microscopic controls that regulate tissue oxygen levels determined by blood flow, oxygen carrying capacity and oxygen release characteristics. Changes in the composition of blood in most cases alter its physical properties, causing changes in blood flow, the distribution of viscosity and pO_2 in the microcirculation. The relationship between these variables can be found by carrying out a mass balance for oxygen at each vessel segment between bifurcations of the circulatory network in order to predict the amount of oxygen present in capillary blood, which determines tissue pO_2.

This analysis shows that capillary pO_2 is determined by a factor proportional to $K = \{\mu/O_2CC \bullet n\}$, where μ is the viscosity of blood, O_2CC is the oxygen carrying capacity of blood, and n is the slope of the oxygen dissociation curve for hemoglobin. When K is large capillary oxygen is low and vice versa. This analysis indicates that in order to obtain normal capillary blood pO_2 both blood viscosity and oxygen carrying capacity must be varied simultaneously, and that higher capillary pO_2 is obtained with steeper oxygen dissociation as a function of pO_2 or left shifted dissociation curves.

Most hemoglobin based blood substitutes utilize molecular hemoglobin in solution that result in blood viscosities that are significantly lower than that of normal blood. Consequently capillary pO_2 is increased, a change that is sensed by the autoregulatory process which reacts by promoting decreased oxygen delivery, i.e., lower blood flow brought about by vasoconstriction. Reduced blood flow decreases microvessel wall shears stress and the release of endothelial shear stress dependant vasodilators further strengthening the vasoconstrictor stimulus, and lowering functional capillary density.

These normal reactions of the organism to lowered blood viscosity in the presence of maintained oxygen carrying capacity can be corrected by increasing viscosity with the addition of plasma expanders of high viscosity, reducing oxygen carrying capacity or manipulating the oxygen dissociation curve. Experimental findings either with a given type of hemoglobin or comparing the behavior of blood substitutes with different K's support this analysis and show that blood viscosity is a preponderant factor in the stabilization of functional capillary density and tissue pO_2.

(Supported in part by USPHS/NHLBI Program Project HL48018 and Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan No. 0708005)

THE EFFECT OF SURFACE MODIFICATION ON THE OXYGEN TRANSPORTING CAPABILITY OF HEMOGLOBIN VESICLE IN VIVO

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To evaluate the oxygen transporting capability of Hemoglobin vesicles (HbV) the physiological responses to 90% exchange transfusions with HbV in anesthetized rat were observed. HbV dispersed in 5% albumin solution was used as sample for the exchange transfusion. The Hb concentration of HbV dispersion was adjusted to 10g/dL. HbV surface-modified with polyoxyethylene (PEG-HbV) was also used in the 90% exchange transfusion. As controls, 5% albumin solution was administered as a non-oxygen carrying fluid and washed rat red blood cells (Hb concentration 10g/dL) as an oxygen carrying fluid. Measurements included mean arterial pressure, arterial and venous blood gas analyses, aortic blood flow and renal cortical and muscle tissue oxygen tensions. At the completion of the exchange transfusion tissue oxygen tensions along with oxygen delivery and oxygen consumption were sustained almost equally well with the HbV dispersion compared to the washed rat red blood cell dispersion, but declined significantly in the albumin solution. These results indicated that the oxygen transporting capability of HbV was almost equivalent to that of rat red blood cells. In the PEG-HbV group, aortic blood flow was sustained higher in comparison to the HbV group. As for blood gas parameters, pH and venous oxygen tension in the PEG-HbV group tended to be higher than those in the HbV group.

IN VITRO DYNAMIC MEASUREMENT OF OXYGEN TRANSFER IN OXYGEN CARRYING SYSTEMS

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A hemoglobin (Hb) vesicle is a phospholipid vesicle which encapsulates concentrated Hb and works as an O_2-carrier. The rate constant of O_2-binding and dissociation on Hb vesicles and intramolecular crosslinked Hb were measured in order to discuss the difference in the cellular and acellular types of O_2-carriers using a stopped flow rapid mixing apparatus.

Equal volumes ($1x10^{-4}$ L) of deoxy-form O_2 carriers ([heme]: $2x10^{-5}$ mol L^{-1}) and buffered solution equilibrated with various O_2 partial pressures (pO_2s) (for O_2-binding rate) or oxy-form O_2 carriers and a buffered solution containing sodium dithionite (for O_2-dissociation rate) were rapidly mixed in a mixing cell at 37 ± 0.2 °C. These reactions were monitored at 415nm (λ_{max} of oxyHb) and 430nm (deoxyHb). The pO_2 of an aqueous phase of the vesicle dispersion was monitored with the triplet lifetime of *meso*-tetraphenylporphinatozinc (ZnTPP) bound to human serum albumin (HSA) by use of a stopped flow flash photolysis system.

The crosslinked Hb was prepared by the reaction of bis(3,5-dibromosalicyl)fumarate with Lys-$\alpha_1$99 and Lys-$\alpha_2$99 of deoxyHb in 0.1 mol L^{-1} HEPES buffer (pH 7.3) for 2 hr at 37 °C [1]. The reaction mixture was heated for 1.5 hr at 75 °C to denature the non-crosslinked Hb. The precipitate was removed by centrifugation (1,900 g, 20 min), and the supernatant was purified by filtration, dialysis, and then ultrafiltration.

The oxygen-binding rate constants of the Hb vesicle and RBC were $3.9x10^5$ and $7.5x10^4$ M^{-1} s^{-1}, respectively [2]. These were significantly smaller than that of crosslinked Hb ($2.0x10^6$ M^{-1} s^{-1}). The Hb concentration in the inner aqueous phases of the encapsulated Hb is 40 g/dL. This result supposed that the oxygenation was limited by the oxygen diffusion because of the high viscosity of concentrated Hb solution. Furthermore, because the oxygen transfer of the Hb vesicle and RBC occurs through the membrane shell, the total surface area is another important parameter used to determine the O_2 binding property.

The relationship between the rate constant of triplet quenching (k_{app}) and pO_2 was clarified to $pO_2 = 4.55x10^{-3} k_{app} - 0.921$. The constant values didn't change in the presence of Hb vesicles. The deoxyHb vesicle dispersion ([heme]= $4x10^{-5}$ mol L^{-1}) was mixed with the HSA-ZnTPP solution ([ZnTPP]= $1x10^{-4}$ mol L^{-1}) containing with O_2 ([O_2]= $1x10^{-4}$ mol L^{-1}). The change of the O_2 concentration shows a biphasic profile. The fast phase supposed facilitated transport by concentrated Hb molecules, and the slow phase indicated the diffusion of on O_2 in the viscous aqueous phase of Hb vesicle. Equilibrium value of pO_2 agrees well with the sum of dissolved oxygen in the Hb vesicle dispersion and released oxygen from Hb vesicles estimated from an oxygen equilibrium curve.

Reference:

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[2] Olson JC (1981) Methods in Enzymology 76, Hemoglobin, Academic Press Inc., New York, pp 631-651

ADVANCES IN THE DEVELOPMENT OF PERFLUBRON EMULSION AS A TEMPORARY OXYGEN CARRIER.

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Perfluorocarbon (PFC) emulsions possess unique properties that make them ideal temporary intravenous oxygen carriers for a variety of clinical indications where tissues may be at risk of hypoxia. The first commercially developed product, *Fluosol*, was developed by the Green Cross Corp. (Japan) over 20 years ago, and represents the only temporary oxygen carrier ever approved by the US Food & Drug Administration (i.e., to preventing myocardial ischemia during prolonged balloon inflation in high risk coronary angioplasty procedures). Some limitations of this first generation product included: 1) the need for frozen storage, 2) the need to thaw and mix with 2 annex solutions prior to use, 3) limited product stability (~8 hours) after reconstitution, and 4) biological side-effects such as occasional complement activation and inhibition of leukocytes which were predominantly due to *Fluosol's* synthetic surfactant, Pluoronic-F68.

Several years ago, Alliance developed an improved second generation, concentrated, stable (2 year shelf life) PFC emulsion based on perflubron (perfluorooctyl bromide) emulsified with egg yolk phospholipid as the only surfactant (median particle diameter ~0.17 μm). This 60% w/v perflubron-based emulsion formulation (AF0144) is currently being tested as an alternative to blood transfusion in surgical patients, as part of a joint development effort with Ortho Biotech and the R.W. Johnson Pharmaceutical Research Institute, subsidiaries of Johnson & Johnson. To date, ~550 subjects have been enrolled in several Phase I and Phase II clinical studies. Extensive safety studies in ~210 healthy volunteers and surgical patients have clearly demonstrated that the current 60% w/v perflubron-based emulsion has no effects on platelet function, (based on *in vitro* aggregation assays), template bleeding times, and coagulation parameters (PT, PTT, fibrinogen). In addition, there was no evidence of any complement activation or immunogenic reactions; no suppression of humoral or cell-mediated immune function; no abnormal changes in either liver, pulmonary, or renal function; no hemodynamic effects or vasoconstriction; and no clinically meaningful effects on blood chemistry. Compared to earlier studies with other PFC emulsion formulations, the incidence and magnitude of the delayed febrile response was substantially reduced. In addition, the decrease in platelet count (<20% from baseline at 3 days) observed in only the high dose group (1.8 g PFC/kg) was less than previously reported for other formulations.

Nonclinical studies in canine models of profound hemodilution, designed to mimic acute surgical anemia and blood loss, have confirmed that perflubron emulsion could prevent tissue hypoxia (brain, heart, gut, liver, and muscle) and preserve myocardial function. Recently, two multicenter, randomized, controlled Phase IIb studies in general surgery (in orthopedic, urological and gynecological patients; n=256) in the USA and in Europe have been completed. Ongoing Phase II studies in cardiac surgery will enroll >100 patients undergoing cardiopulmonary bypass. Pivotal Phase III studies to demonstrate efficacy of perflubron emulsion as a temporary oxygen carrier are expected to begin in the fall of 1997.

Oxygen carrying capacity and oxygen supply rate of artificial oxygen carrier, Neo Red Cell (NRC)

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Oxygen carrying capacity and oxygen supply rate of artificial oxygen carrier, Neo Red Cell (NRC), were estimated by continuous measurements of dissolved oxygen concentration by an oxygen electrode in a sealed vessel containing 55mL of hepatocyte ($1x10^6$ cells/mL) at 37℃. The specific oxygen uptake rate (Q_{O_2}) of freshly harvested Wistar rat hepatocyte was estimated to be $2.7x10^{-9}$ mmol-O_2/cell/h and was found constant in the dissolved oxygen concentration range 0.03 - 0.19 mmol/L. NRC (final hemoglobin concentration was 5w/v%) was added to the hepatocyte suspension and oxygen supply rate (OSR) of NRC was calculated as follows;

$$OSR = r_{O_2} - dc/dt$$

Where, r_{O_2} is the oxygen uptake rate of hepatocyte which is the product of Q_{O_2} and cell density (1×10^6 /mL), and dc/dt is the change in dissolved oxygen concentration with time which is calculated from the trace of dissolved oxygen concentration in the sealed vessel.

The OSR of NRC was increased with the decline of dissolved oxygen. The OSR of NRC can be considered to depend on the oxygen affinity of NRC. In a cell suspension without NRC, hepatocytes consumed dissolved oxygen from 0.19 to 0.03 mmol/L within 3.5 minutes. The NRC-containing suspension extended the oxygen supply to hepatocytes over 25 minutes.

These results indicate that the oxygen supply from NRC may sustain the high-density culture of mammalian cells.

EVALUATION OF THE OXYGEN TRANSPORTING CAPABILITY OF ENCAPSULATED HEMOGLOBINS IN ANIMAL MODELS

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The oxygen transporting capability of encapsulated hemoglobins were evaluated in animal models. Two types of encapsulated hemoglobins were used in the experiments. Hemoglobin vesicle (HbV) and Neo Red Cells (NRC). HbV has a particle size of approximately 240nm, hemoglobin concentration is 10g/dl and its P50 is controlled to 32 Torr.
NRC has a particle size of approximately 200nm, hemoglobin concentration is 5g/dl and its P_{50} is controlled to 45 Torr. The protocols employed were exchange transfusions, hemorrhagic shock resuscitations and extracorporeal circulation. The measured parameters of oxygen transport included arterial and venous blood gas analyses, oxygen delivery, oxygen consumption and tissue oxygen tensions. The measurements were compared between the administration of non oxygen carrying fluids, encapsulated hemoglobins or washed red blood cells, in each protocol. Encapsulated hemoglobins were able to maintain the parameters of oxygen transport almost as well as red blood cells suggesting that their oxygen transporting capability were equivalent to red blood cells.

PROPERTIES AND OXYGEN BINDING ABILITY OF ALBUMIN/TETRAPHENYL-PORPHYRINATO-IRON(II) DERIVATIVE COMPLEXES

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We have recently found that a 1:1 complex of synthetic porphyrinato-iron(II) derivative (Fe(II)P) and human serum albumin gave a stable oxygen adduct in an aqueous phase. Since then, the preparation procedure has been significantly improved to prepare more concentrated solutions of the complex and to bind a greater number of Fe(II)Ps into one HSA molecule so as to make the oxygen transporting ability comparable to hemoglobin. This paper reports on a new preparation procedure of HSA-Fe(II)P complexes which bind one, four, and eight Fe(II)P molecules, and also describes the characteristics and oxygen-binding properties of the complexes.

The L-ascorbic acid aqueous solution (100 μL) was added to a Fe(III)P ethanol solution ([Fe(III)P]=0.15 mM) under CO atmosphere in order to reduce Fe(III)P. A 200 mL aliquot of the Fe(II)P(CO) solution was then mixed with 400 mL of the HSA solution (5 wt %), giving an HSA/Fe(II)P (molar ratio = 1:4). The obtained red transparent mixture (600 mL) was evaporated, dialyzed, and further concentrated by ultrafiltration (cut off Mw: 50,000). The HSA/Fe(II)P (molar ratio = 1:8) solution was also prepared using the same procedure. In this case, the HSA concentration was decreased to half ([HSA] = 9.4 mM), and the final Fe(II)P concentration was adjusted to 6 mM.

The HSA/Fe(II)P complex provided a reversible and relatively stable oxygen adduct under physiological conditions (pH 7.4, 37 °C). The half-life of the oxygen adduct ($t_{1/2}$) was 1 h at 37°C in air atmosphere. HSA bound Fe(II)TpivPP, so called "picket-fence heme having no axial base, was also able to form oxygen adduct only when a 20-fold excess mol of 1,2-dimethylimidazole was existed, the $t_{1/2}$ was, however, very short (ca. 10 min at 37°C).

The oxygen affinity ($P_{1/2}(O_2)$) and oxygen transporting efficiency (OTE) of HSA/Fe(II)P at 37°C were 30 Torr and 22%, respectively. Furthermore, the oxygen-binding and dissociation rate constants (k_{on}, k_{off}) are extremely high compared with those of red blood cells. The HSA/Fe(II)P (molar ratio = 1:8) can transport ca. 3.4 mL/dL of oxygen under physiological conditions, corresponding to about sixty percent of the oxygen transporting amount of human blood.

The binding site of Fe(II)P to HSA were determined using several ligands, of which binding sites have been known. The maximum number of Fe(II)P bound to HSA was eight. When one of the Fe(II)P binding site was occupied by the ligand, the number should be reduced to seven. The binding sites of Fe(II)P thus determined were the primary binding sites of hemin and bilirubin.

Reference:
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A SEVENTY TWO HOURS HYPOTHERMIC INTESTINAL PRESERVATION STUDY USING A NEW PERFLUOROCARBON EMULSION.

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Aim : Quality of long term intestinal preservation remains disappointing. Increasing oxygenation during preservation has the potential to fulfill the remaining metabolic demand of the organ in hypothermia. We have investigated the effect of adding an effective fluorocarbon emulsion, with a high oxygen carrying capacity, to the Univ. of Wisconsin solution.
Methods : Small bowel grafts harvested from Lewis rats were preserved at 4°C for 12-72h, followed by histological assessment and measurements of tissue antioxidant potential. The concentrated perfluorocarbon (FC) emulsion consisted of perfluorooctyl bromide (90% w/v, 47% v/v) emulsified with egg yolk phospholipids (2% w/v) and stabilized with mixed fluorocarbon-hydrocarbon molecular dowels (F6H10; 1.4% w/v) (droplets of ca 0.2 μm, pH 6.9, viscosity 20.2 cp at 0.73 s-1). This emulsion was stable when diluted with the organ preservation fluids. Four groups were defined depending on the type of vascular and luminal flush, and storage medium : (1) UW/UW : UW flush and storage in UW; (2) UW/FC : UW flush and FC storage; (3) FU/FU : flush with FC diluted 2 times with UW (FU) and storage in FU; (4) FU/FU±O_2 : flush with FU and storage in oxygenated FU. Histological grading was performed using a villi score (0-4) based on the adhesion of the villus epithelium to the lamina propria and on villus sloughing, and a crypt score (0-4) based on the adhesion of crypt cells to the basal membrane. Antioxidant potential of intestinal tissue was estimated by spectrophotometric measurement of the total thiol functions (-SH) and enzymatic activities of glutathione-peroxidase (GSH-P), superoxide dismutase (SOD) and catalase (Cat), for groups 1, 3 and 4. Supernatants of homogenized tissues were used after centrifugation at 27,000G.
Results : Increasing preservation injuries are observed with time (table 1). Addition of the FC emulsion to the vascular flush improved preservation during the first 24h ($p<0.01$) significantly. The efficacy of intestinal storage in the pure emulsion appeared superior to UW storage at 24h ($p<0.01$). Oxygenation of the storage medium with 100% O_2 yielded even superior results at the 12h ($p<0.01$) and 24h ($p<0.001$) time points. As preservation is extended, differences among the 4 groups progressively reach non significance.

Preservation duration	UW/UW group 1	UW/FC group 2	FU/FU group 3	FU/FU±O_2 group 4
0h	7.5 ± 0.0	7.5 ± 0.0	8.0 ± 0.0	7.5 ± 0.0
12h	5.8 ± 0.3	5.3 ± 0.3	6.2 ± 0.3	7.1 ± 0.2
24h	4.4 ± 0.4	5.8 ± 0.1	5.8 ± 0.7	6.4 ± 0.2
48h	3.8 ± 0.8	4.4 ± 1.1	2.9 ± 0.8	4.9 ± 0.5
72h	2.4 ± 0.6	2.6 ± 1.1	3.3 ± 1.7	1.3 ± 0.3

Table 1 : Evolution of histological score (0-8) during 72h preservation at +4°C (mean ±SEM). n=5 in each group; at each time point, 5 histological samples were blindly assessed

For SOD and Cat, enzymatic activities remained unchanged after 24 and 48 h in all groups. After 72h their activities increased in groups 3 and 4, while SOD decreased in group 1 ($p<0.05$). Total -SH progressively decreased with preservation time in group 1 ($p<0.05$, ANOVA test), and remained unchanged in groups 3 and 4. GSH-P did not change in group 1, but progressively increased at 24 and 48h in groups 3 and 4 ($p<0.01$).
Conclusions : The addition of a perfluorocarbon emulsion to the preservation flush or storage medium appeared devoid of deleterious effects. On the contrary, the increase of oxygen concentration in the perfusion flush or storage medium proved to ameliorate the preservation status and to better protect the antioxidant potential of the small bowel. These results also establish that FC emulsions are without specific cytotoxicity, confirming our previous data obtained on endothelial cells.

VIABILITY OF THE RAT ILEUM PERFUSED BY VARIOUS OXYGEN CARRIERS

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Background: In vivo physiological studies of the intestine are limited by invasiveness of investigation methods. In vitro intestinal preparations are more practical but depend critically on oxygen delivery. As oxygenated Krebs-Ringer solutions do not secure tissue viability, arterial perfusions with an oxygen carrier are required. In a long term study of small intestinal rhythmicity, we used 3 oygen carriers: fresh bovine erythrocytes suspended in Krebs solution (ERY), Oxypherol® (OPH) and Perflubron (PER) emulsions. Their effects on viability and functional integrity of in vitro ileum of the rat are reported here.
Methods: Segments of terminal ileum (4-6 cm) were arterially perfused with solutions containing the oxygen carrier (n = 12 for each condition) and luminally with saline. Arterial pressure, oral (Por) and aboral (Pab) luminal pressures, as well as video images of the segment were recorded simultaneously. Video image analysis was used to characterize low amplitude contractile events and to detect early functional damage due to hypoxia, such as microembolisations, mucosal desquamation and carrier leakage into the lumen. Variations of arterial pressure (ΔPart) were used as criteria of perfusion and mesenteric vasoreactivity.
Results: As previously described*, the ileum generated two motor rhythms, namely, propulsive neurogenic (NC) and myogenic contractions (MC). Parameters characterizing these rhythms, time at which occurred the first damage, as well as fluctuations of arterial pressure are listed in the table (data are medians and IQ intervals). The new perflubron (perfluorooctyl bromide) emulsion contained 90% w/v (47% v/v) of the fluorocarbon, 2% w/v of egg yolk phospholipids as the emulsifier and 1.4% w/v of $C_6F_{13}C_{10}H_{21}$, a "molecular dowel", as the stabilizer. Emulsification was achieved by microfluidization and the product was heat sterilized. Average particle size was *ca* 150 nm, pH was 6.9 and viscosity was 20.2 cp at 0.73 s-1 **. Prior to use the emulsion was combined with a saline solution containing albumin and glucose. Final fluorocarbon concentration was 27% w/v. The added salt solution had only little effect on emulsion characteristics.

	Neurogenic Contractions			Myogenic Contractions		Time to Damage	ΔPart
	Freq (c/min)	Por (hPa)	Pab (hPa)	Freq (c/min)	P (hPa)	(min)	(mm Hg)
ERY	0.12 (0.36)	°11.9 (18.6)	°2.9 (10.5)	23.7 (2.5)	0.30 (0.17)	55 (21.0)	n.d.
OPH	0.31 (0.11)	°21.1 (9.1)	°13.5 (4.3)	24.0 (0.5)	0.26 (0.15)	56 (38.2)	10(16)
PER	0.28 (0.11)	17.3 (8.0)	15.5 (4.1)	24.0 (1.9)	0.29 (0.50)	*85 (41.5)	*110(81)

(°p<0.05, oro-aboral comparison, Wilcoxon; *p<0.05, Mann-Whitney; n.d. not determined)

With ERY, NC frequency was not changed (but its variance was increased, F-test: p<0.01) and NC amplitudes were smaller (p<0.05). With ERY and OPH, large differences between oral and aboral pressures were found and the first mucosal damage appeared already within 1 hour. These results suggest that the enteric nervous system underwent mild hypoxic conditions leading to disturbances of excitatory neurotransmission and proximo-distal coordination. The parameters of MC were independent of the carrier used, which confirms that visceral smooth muscle can withstand mild hypoxia. In addition, when OPH was used, endothelium dependent mesenteric vasoregulation was missing. This could be explained by damage to endothelium by the Pluronic used as emulsifier.

Conclusion: The preservation of the intestinal neurogenic motor pattern, known as the most sensitive to hypoxia, was significantly better with perfusion using a new formula of Perflubron emulsion prepared with natural phospholipids and stabilized with a "molecular dowel".

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*Bercik *et al.*, Gastroenterology **106**, 649(1994). **Riess *et al.*, Adv. Exp. Biol. Med. **345**, 227(1994).

ALTERATIONS OF THE STRUCTURE AND SIDE REACTION OF RERFLUOROCARBON EMULSIONS AT STORAGE

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Using approaches elaborated by us we studied the physico-chemical parameters of perfluorocarbon (PFC) emulsions, that describe the peculiarity of alteration of their structure: average diameter of particles **d** and particle size distribution (these parameters characterize the structure of PFC-phase); the refractive index of PFC emulsion particles which characterizes the macrostructure; index of PFC emulsion interaction with blood serum - K_τ, whose changes characterize microstructure alterations. At the same time we evaluated using neufropenic index I_n also the side anaphylactoid reaction induced by PFC emulsions in their intravenous administration to rabbits. For PFC emulsions that are biocompatible the values of I_n should not be more than 2-3.

The structure of PFC-phase, microstructure and I_n of frozen PFC emulsion prepared using hydrophilic surfactant procsanol-268 (pluronic F-68) did not change during some monthes of storage.After thawing and one month of storage at 4°C **d** did not change, but indices K_τ and I_n increased. In another words, breaking of the microstructure of PFC emulsiuons occurs simultaneously with deterioration of their biocompatibility.

The structure alteration and side reaction of perfluorodecalin (PDF) - based emulsions with addition of lipophilic PFC component were also studied. The emulsions were prepared using hydrophobic surfactant,egg yolk phospholipids (EYP). The structure of PFD phase and the microstructure of these emulsions changed insignificantly during 1-6 monthes of storage at 4°C. Values of I_n ranged between 1 and 3 during this period of study. The results obtained testify that emulsions PFD/EYP stored at 4°C have their structure and biocompatibility unchanged.

Macrostructure of studied emulsions did not changed in all cases.

Thus, study of the alteration ,at storage,of PFC emulsion structure using the parameters proposed by us increase the reliability of data about their stability and biocompatibility.

RECOMBINANT HEMOGLOBINS FOR OXYGEN-CARRYING THERAPEUTICS: CONTROL OF NO SCAVENGING AND O_2 BINDING.

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A series of recombinant hemoglobins have been made that vary in oxygen binding parameters and reactivity with nitric oxide. The goal of this work is to produce hemoglobins that are free of NO-related side effects but retain efficacy as oxygen-delivering pharmaceuticals. To achieve this goal, we sought to reduce NO scavenging as much as possible while maintaining sufficient oxygen kinetics and equilibrium. Our strategy was substitution of distal heme pocket amino acids to sterically limit the access of NO (Eich *et al.*, 1996 *Biochemistry* 35, 6979-6983). Recombinant hemoglobin variants consisting of substituted alphas or betas (paired with wild-type partner subunits) were tested *in vitro* for rates of reaction with nitric oxide and rates of oxyhemoglobin dissociation. Selected di-alpha and beta subunits were then combined to generate fully substituted hemoglobin tetramers that could be tested in animal models of side effects and efficacy. Additional substitutions, previously shown to affect the allosteric equilibrium of hemoglobin, were incorporated into the tetrameric constructs to alter the P_{50}. These new hemoglobins were tested *in vitro* and a subset were selected for animal experiments based on the kinetic and equilibrium parameters. We found that the rates of oxygen dissociation were not correlated with the rates of reaction with NO. O_2 kinetics and equilibria were manipulated by varying the degree of steric hindrance near the iron atom, by varying the strength of hydrogen bonding between the E7 residue and the bound oxygen, and by changing the position of the allosteric equilibrium. The latter method is independent of NO reaction rate. While changing the R/T equilibrium significantly alters oxygen binding, there is no effect on the rate of reaction of NO with oxyhemoglobin. Varying steric hindrance and polarity in the distal pocket affects both processes. The rate constants for NO scavenging by the new oxyhemoglobins ranged from 1-60 $\mu M^{-1}s^{-1}$. We have shown previously that the *in vivo* pressor response to extracellular hemoglobins is roughly proportional to the rate of NO scavenging, but not correlated with O_2 affinity. Even a modest decrease in the rate of NO scavenging resulted in a demonstrably lower pressor effect. Here we show that the total peripheral resistance measured in rats is also dependent on the rate of NO scavenging. Furthermore, several of these novel hemoglobins have acceptable oxygen kinetics and equilibria and cause little or no peripheral vasoconstriction.

CULTURE OF CELLS AT PERFLUOROCARBON-AQUEOUS INTERFACES

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A requirement in many facets of biotechnology, involves systems for maintaining eukaryotic cells under static conditions, but with adequate respiratory gas supplies. This is especially true for cells recovered from cryo-storage which are vunerable, in the short-term, to physical damage. One such approach involves the culture of cells at an interface between inert, oxygen-gassed perfluorocarbon (PFC) liquid (e.g. *Flutec*® PP6, BNFL Fluorochemicals Ltd., U.K.) overlaid with liquid or semi-solidified media. Such systems have been used previously for human cells (e.g. fibroblasts, retinoblastomas, HeLa cells), which divided normally on a protein monolayer precipitated at the interface. In the present study, protoplasts (wall-less cells) of higher plants (e.g. *Petunia hybrida*, *Oryza sativa*) were cultured for up to 21 days under such conditions. For plant cells, supplementation of aqueous medium with 0.01% (w/v) of *Pluronic*® F-68 not only lowered interfacial tension, but further enhanced cellular mitotic activity over that stimulated by oxygenated PFC. For totipotent plant protoplasts, such as those of *O. sativa*, culture with oxygenated PFC increased biomass and, significantly, further enhanced the regeneration of protoplast-derived shoots leading to phenotypically- and genotypically-normal plants. A further gas delivery option is the addition, to the aqueous phase, of haemoglobin solution (e.g. *Erythrogen*™) to further increase cell oxygenation. The advantages of such systems include (1) ease of sterilisation of the PFC by autoclaving, (2) the recycleability and, hence, recovery of the PFC, thereby offsetting high initial costs, (3) the ability to aspirate cells from the interface, and (4) their applicability for anaerobic (e.g. CO_2-gassed) cell systems.

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ENHANCED MITOSIS IN CULTURED PROTOPLASTS: BENEFICIAL EFFECTS OF OXYGENATED PERFLUOROCARBON AND HAEMOGLOBIN

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Mitotic division of protoplasts (wall-less cells) can be enhanced theoretically by culture in the presence of oxygenated perfluorocarbon (PFC) and/or supplementation of culture medium with haemoglobin solution. The present study has examined such effects on the culture of cell suspension-derived protoplasts of *Petunia hybrida* cv. Comanche in (1) liquid medium overlaying oxygenated (10 mbar, 15 min) PFC (*Flutec*® PP6, BNFL Fluorochemicals, U.K.), (2) medium containing 1:50 (v/v) of *Erythrogen*™ (Biorelease Corporation, U.S.A.), or (3) medium containing 1:50 (v/v) of *Erythrogen*™ overlaying oxygenated PFC. The mean initial plating effiency (IPE) after 10 days of protoplasts cultured with oxygenated PFC (20.3 $\pm$ 1.5%; n = 5 throughout) was significantly greater ($P < 0.05$) than control (9.6 $\pm$ 0.6%). Similarly, a significantly ($P < 0.05$) greater mean IPE of protoplasts cultured in medium containing *Erythrogen*™ was observed (17.3 $\pm$ 1.1%). There were no significant differences in IPE between oxygenated PFC and *Erythrogen*™ treatments. When the latter two treatments were combined, the mean IPE (23.0 $\pm$ 0.9%) was also significantly ($P < 0.05$) greater than for untreated controls or cultures supplemented with *Erythrogen*™. There were also no significant differences with respect to mean IPE of protoplasts cultured with oxygenated PFC and *Erythrogen*™ in combination and protoplasts cultured with oxygenated PFC alone. These results indicate that oxygenated PFC and *Erythrogen*™ provide viable options for enhancing oxygen supply to eukaryotic cells *in vitro*. However, the recoverability and hence, recycleability of PFCs make them a commercially attractive option, despite a high initial investment cost.

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HAEMOGLOBIN (*ERYTHROGEN*™)-ENHANCED POST-THAW GROWTH OF CRYOPRESERVED CELLS

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A key requirement for maximising the recovery of cryopreserved cells is the optimisation of post-thaw culture conditions, notably respiratory gas regulation. One novel approach to facilitate oxygen supply, which has not been evaluated previously for cryopreserved cell systems, is the supplementation with haemoglobin (Hb) solution of post-cryopreservation culture medium. Native and, to a greater extent, chemically-modified human and bovine Hbs, have been evaluated *in vivo* as vehicles for respiratory gas transport and also provide an option for gas regulation *in vitro*. In the present study, cells of *Oryza sativa*, initiated from embryogenic calli derived from mature seed scutella, were employed to assess the beneficial effects of a commercial bovine Hb solution, *Erythrogen*™ (Biorelease Corporation, U.S.A., supplied through TCS Biologicals, U.K.), as a culture medium supplement to enhance post-thaw mitotic competence following short-term (30 days) cell cryopreservation. Compared to controls, supplementation of medium with *Erythrogen*™ (1:50-1:500 v:v) increased mean cell absorbance (viability) following triphenyl tetrazolium chloride reduction (8 days post-thaw), by up to 60% ($P < 0.05$). Specific concentrations of *Erythrogen*™ (1:50 or 1:100 v:v) enhanced mitotic activity of the cell populations, with the mean biomass accumulation (24 days post-thaw) increasing by up to 22% ($P < 0.05$) over control. Cryopreservation of cells is, by definition, an underpin technology ensuring germplasm conservation for subsequent biotechnological exploitation. To this latter end, the suitability of *Erythrogen*™-treated cells was assessed in the context of protoplast production. Protoplast yields, from such cells, were comparable to those of unfrozen control cultures.

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NOVEL FLUOROCARBON EMULSIONS AS BLOOD SUBSTITUTES

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Emulsions of perfluorocarbons (PFCs) have been evaluated clinically as respiratory gas carriers and diagnostic contrast imaging agents. Interest is currently focused on the development of "second-" and "third-generation" emulsions with improved stability characteristics and, in some cases, increased PFC content giving superior oxygen-carrying capacity. A novel series of experimental PFC emulsions, based on the cyclic compound, perfluorodecalin (FDC), stabilised with up to 2.5% (w/v) of lecithin (*Lipoid*® E100, Lipoid GmbH) have been produced. In some cases 0.1% (w/v) of a novel, amphiphilic fluoro-surfactant, $C_8F_{17}C_2H_4NHC(O)(CH_2CH_2O)_2Me$ (P6), a fluorinated polyol derivative, was added to enhance emulsion stability. Some formulations also contained 1.0% (w/v) of perfluorodimorpholinopropane to retard droplet growth through molecular diffusion (Ostwald Ripening). The emulsions were prepared by homogenisation and were steam sterilisable (121°C, 2 atm, 20 min) with no significant change in mean droplet diameter (*ca.* 0.2 μm); such emulsions were stable for up to 200 days (37°C). Injection of male Wistar rats (221-279 g; n = 30) with 7.5 ml kg^{-1} body weight of emulsion produced significant ($P < 0.05$), transient increases in both mean liver and spleen weights, compared to saline-treated controls, that were consistent with previous related observations. One emulsion inhibited phorbol 12-myristate 13-acetate (PMA)-stimulated, *Luminol*®-enhanced, chemiluminescence of human neutrophils *in vitro*, suggesting possible applications in ischaemic tissues for suppressing leucocyte-mediated inflammation. The P6 fluoro-surfactant also inhibited spontaneous platelet aggregation in hirudin-anticoagulated human blood *in vitro*, indicating possible applications as an anti-thrombotic agent which requires further study.

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PLURONIC® F-68 INHIBITS AGONIST-INDUCED PLATELET AGGREGATION IN HUMAN WHOLE BLOOD *IN VITRO*

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The non-ionic, co-polymer surfactant, Pluronic F-68 (poloxamer 188; PF-68), commonly-used as a stabiliser of perfluorochemical emulsions for *in vivo* oxygen delivery, can inhibit spontaneous platelet aggregation in human whole blood [1], but the mechanism of this effect remains obscure. PF-68 may be valuable as an anti-thrombotic agent, but its interaction with known platelet aggregation agonists needs further study. Therefore, the effects have been examined of PF-68 (0.04% w/v) on platelet aggregation in hirudin (50 $\mu g\ ml^{-1}$)-anticoagulated, human whole blood *in vitro* in response to the following aggregation agonists: (i) adenosine di-phosphate (ADP; 0.3, 1.0 or 3.0 μM), (ii) platelet activating factor (PAF; 0.75, 1.5 or 3.0 μM), (iii) phorbol 12-myristate 13-acetate (PMA; 0.05, 0.1 or 0.15 $\mu g\ ml^{-1}$), (iv) collagen (0.125, 0.25 or 0.5 $\mu g\ ml^{-1}$) or (v) ristocetin (0.3, 0.6 or 1.2 $\mu g\ ml^{-1}$). PF-68 significantly ($P < 0.05$) inhibited platelet aggregation that followed the addition of all agonists at their lowest concentration tested. Interestingly, PF-68 also accelerated of the rate of the platelet dis-aggregation that followed the addition of either ADP or PAF to PF-68-treated blood. PF-68 had markedly less pronounced inhibitory effects on the platelet aggregation that occurred in response to 0.15 $\mu g\ ml^{-1}$ PMA, where the mean % aggregation after 8 min was 67% of control. PF-68 did not alter platelet aggregation in blood treated with 0.25 or 0.5 $\mu g/ml$ of collagen. These results show that PF-68 can inhibit agonist-induced platelet aggregation in human blood *in vitro*. The ability of PF-68 to inhibit platelet aggregation in the presence of weak agonists, such as ADP, and the lower doses of the more potent agonists, PAF, PMA, collagen and ristocetin, which act through different receptor pathways, suggests a non-specific mechanism of action of the surfactant involving cell surface adsorption.

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EVALUATION OF COMMERCIAL AND PURIFIED *PLURONIC®* F-68 IN A HUMAN BLOOD NEUTROPHIL BIOASSAY

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Pluronic F-68 (poloxamer 188; PF-68) has been used as an emulsifier in "first-generation", fluorocarbon emulsion-based *in vivo* oxygen carriers, principally *Fluosol®*. PF-68 was implicated as the constituent of *Fluosol®* responsible for adverse, albeit transient, effects on blood leukocytes (PMNL) and complement activation [1]. Paradoxically, such effects of PF-68 may have therapeutic advantages in ischaemic tissues for suppressing PMNL-mediated reperfusion injury. Since commercial (COMM) PF-68 can contain variable amounts of undisclosed impurities, the effects of various PF-68 fractions have been evaluated in a human blood PMNL bioassay *in vitro*. Citrated blood (4.5 ml) was pre-incubated (30 min) in a water bath (37°C). Fifty μl of blood was added to 10 μl of saline (0.9% w/v NaCl), COMM PF-68 (4.0% w/v) or purified (PUR) PF-68 fractions prepared by passage through silica gel resin (SGR) or by supercritical fluid fractionation (SFF), followed by drying under vacuum (80°C); samples were re-incubated for a further 2 min. Fifty μl of sample was added to PBS (1.0 ml) containing *Luminol* (100 μg ml^{-1}) and incubated for a further 5 min (37°C). Twenty μl of phorbol 12-myristate 13-acetate (PMA; 100 μg ml^{-1}) was added to each sample and the chemiluminescence (CHML) recorded every 2 min for 20 min. The mean ($\pm$ s.d., n = 3) total CHML, as assessed by the area under the curve, following stimulation of neutrophils with PMA in saline controls was 190 $\pm$ 3 mV x min. COMM F-68 inhibited CHML by up to 26% ($P < 0.05$). In contrast, partially PUR PF-68 prepared by SGR or SFF stimulated CHML by up to 53% over control ($P< 0.05$). The total CHML with PUR PF-68 prepared by SFF followed by SGR was not significantly different to that produced by saline. The present results, showing that a highly purified PF-68 fraction has minimal effects in a human neutrophil bioassay, reinforce previous suggestions that trace impurities in COMM grade preparations of the surfactant are responsible for their adverse biological effects.

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DCLHb, A HEMOGLOBIN-BASED OXYGEN CARRIER: MANUFACTURE AND CHARACTERIZATION FOR USE IN CLINICAL STUDIES

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Human hemoglobin must be modified if its formulations are to be suitable for clinical use. One modified human hemoglobin that is now in clinical trials is DCLHb, a hemoglobin that is covalently crosslinked between the alpha chains by a bis(fumaramide) bridge. DCLHb is manufactured in a five-stage process that includes the following steps: (1) hemoglobin (Hb) isolation from human red cells that have been tested and found suitable for transfusion, followed by Hb purification; (2) intramolecular crosslinking of deoxyHb by bis(3,5-dibromosalicyl) fumarate (DBBF) in the presence of tripolyphosphate (a DPG analog); (3) heat-treatment; (4) filtration and diafiltration into a physiological electrolyte solution; and (5) packaging and freezing for long-term storage. Steps 1 and 3 of this process effect virus removal and inactivation, respectively. DCLHb is tested for release using approximately twenty assays. Additional details regarding the manufacture and release testing of DCLHb will be provided. In addition, the storage stability of DCLHb has been studied, and DCLHb has been characterized using techniques such as peptide mapping, electronic circular dichroism, and fluorescence spectroscopy. Results of these characterizations will be presented. In summary, the use of validated manufacturing processes and release tests has enabled the production of over thirty batches of DCLHb that were suitable for use in clinical trials.

DCLHb, A WELL-CHARACTERIZED HEMOGLOBIN

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Diaspirin crosslinked hemoglobin (DCLHb), a hemoglobin-based oxygen carrier having therapeutic potential, is in Phase III clinical trials. DCLHb is an intramolecularly crosslinked human hemoglobin in which a fumaryl bridge covalently links the lysine 99 residues of the α-subunits. We have investigated the effect of crosslinking on the structure and function of DCLHb in comparison to human hemoglobin. The oxygen affinity and high degree of cooperativity of oxygen binding exhibited by DCLHb were similar to the corresponding properties of hemoglobin in the red cell *in vivo*. Size exclusion chromatography demonstrated that more than 98% of the product is intramolecularly crosslinked. Reversed phase HPLC revealed the globin of DCLHb was composed of only two types of constituent peptide chains: β protein chains and αα crosslinked protein chains. The exact site of crosslinking, which x-ray analysis indicated was between Lys 99α1 and Lys 99α2, was confirmed by mass spectrometry. Circular dichroism spectra in the far-UV showed no differences in the secondary structure between DCLHb (70% α-helix) and human hemoglobin. Front-face fluorescence spectroscopy showed that the tertiary structure of DCLHb was also similar to human hemoglobin. UV Resonance Raman studies show the H-bond between Trp β37 and Asp α94 to be unaffected by crosslinking. This quaternary contact is significant to Hb ligand binding since it forms part of the α1-β2 interface, a key locus of the conformation transition that occurs in cooperative ligand binding. All of these results support the conclusion that the structural and functional integrity of human hemoglobin has been preserved in DCLHb.

EVALUATION OF THE IMMUNOGENICITY OF DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb).

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DCLHb, a hemoglobin-based oxygen carrier having therapeutic potential, is in Phase III clinical trials. DCLHb is an intramolecularly crosslinked human hemoglobin that overcomes several shortcomings of stroma-free hemoglobin (SFHb), including the inability of SFHb to unload oxygen to tissues and the nephrotoxicity that results from extensive renal excretion of dimeric subunits of SFHb. In addition to its useful oxygen-carrying properties, DCLHb appears to function as an active oncotic and pharmacologic agent, and may be capable of restoring blood pressure, oxygen delivery and circulation following acute hypotension after insults such as hypovolemic shock. As a part of the extensive safety evaluation of DCLHb, we have investigated the immunogenicity of DCLHb. To determine if DCLHb is immunogenic, sera from patients infused with DCLHb were collected 3-8 weeks post-infusion and analyzed for IgG antibodies specific to DCLHb. To measure these antibodies, ELISA and western blot assays were developed and validated. Experiments directed to the development and validation of both immunoassays have focused on specificity and sensitivity. Both the ELISA and the western blot assays were specific to DCLHb and SFHb. The cross-reactivity of the DCLHb antibodies to SFHb was expected, since the positive control for DCLHb antibodies is a polyclonal antibody which was raised in rhesus monkeys. Additionally, SFHb and DCLHb are structurally similar, with exception that DCLHb has an internal, covalent crosslink between two lysine 99 residues of the α subunits. For the ELISA test, the response of monkey anti-sera to DCLHb coated wells was the positive control and that of normal human sera to carbonate buffer coated wells was the negative control, and a "positive/negative cut-off" value of 1.4 was determined by statistical analysis of the measured optical densities. This value is used to report DCLHb antibody test results as positive or negative for individual patients. Thus, if a patient sample has an optical density ratio greater than 1.4, the sample is considered reactive for DCLHb antibodies. A ratio of less than 1.4 is considered negative for DCLHb antibodies. Western blot assay is used as a confirmatory assay for an ELISA reactive sample. To date, more than 200 patients treated with DCLHb have been screened by ELISA and have shown no evidence of antibodies to DCLHb. These results suggest that DCLHb is non-immunogenic.

HEMODYNAMIC RESPONSE FOLLOWING ADMINISTRATION OF HEMOLINK™ IN CONSCIOUS AND ANESTHETIZED SPONTANEOUSLY HYPERTENSIVE RATS

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Hemolink™ is a periodate ring-opened raffinose crosslinked and polymerized hemoglobin solution prepared from human red blood cells. Pre-clinical and human trials demonstrated that Hemolink™ had no significant toxicity. However, a mild pressor effect was observed in some animal models during pre-clinical studies as well as in healthy human volunteers during a Phase I safety trial. To assess the hemodynamic effects of Hemolink™ in untreated hypertensive and treated hypertensive subjects, further investigations in animal models were undertaken. This study evaluated the hemodynamic response to Hemolink™ in both conscious and in anesthetized (isoflurane/Somnotol) spontaneously hypertensive rats (SHR rats). Normotensive rats from the base stock (WKY rats) were used for comparison. The results are shown in the Table.

	MAP (mmHg)				Heart Rate (bpm)			
	conscious		anesthetized		conscious		anesthetized	
Time	SHR (n=11)	WKY (n=9)	SHR (n=13)	WKY (n=13)	SHR (n=11)	WKY (n=9)	SHR (n=13)	WKY (n=13)
pre-infusion	117±4	85±2	156±5	96±2	438±11	455±13	392±10	390±10
post-infusion 5 min	144±3	97±2	170±6	108±4	394±11	386±19	359±8	347±7
10 min	145±4	99±3	168±5	110±4	376±12	371±21	347±6	347±7
15 min	146±3	101±1	163±6	110±4	369±13	349±12	344±6	348±9
20 min	149±3	100±1	162±6	110±4	358±14	335±13	346±7	359±9
25 min	151±4	99±2	160±5	108±4	360±18	323±8	351±9	363±10
30 min	151±4	99±2	156±6	106±4	368±16	323±9	359±10	367±10

Conscious SHR rats had a significantly higher baseline mean arterial blood pressure (MAP) than WKY rats. Following a 10% topload infusion of Hemolink™, MAP increased 25-35 mmHg (20-30%) in SHR rats and 12-15 mmHg (14-20%) in WKY rats. The difference was statistically significant ($P<0.01$). Baseline heart rates were not significantly different between the two conscious groups. Following the administration of Hemolink™, heart rate decreased 50-80 bpm (11-20%) in SHR rats and 70-130 bpm (15-30%) in WKY rats. The decrease in HR was significantly less in SHR rats than in WKY rats ($P<0.01$). In anesthetized animals, the baseline MAP was significantly higher in the SHR group than in the WKY group. Baseline heart rates were not different between the two groups. Following a 10% topload infusion of Hemolink™, MAP increased transiently up to 15 mmHg (10% for SHR; 15% for WKY) and HR decreased 40-50 bpm (11-13%) in both groups. This study suggests that Hemolink™-induced hemodynamic response is different in conscious SHR rats and WKY rats. When Hemolink™ is administered during isoflurane/Somnotol anesthesia, both the degree of the hemodynamic response in each group and the difference in the response between the two rat strains are diminished.

HEMOLINK™ INDUCED EFFECTS ON THE INTESTINAL MOTOR FUNCTION AND POSSIBLE TREATMENTS

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Purpose: Hemolink™, a human hemoglobin solution crosslinked and polymerized with periodate ring-opened raffinose, inhibits lower esophageal sphincter relaxation and increases esophageal peristaltic velocity in both the opossum and cat, an effect attributed to the hemoglobin's ability to bind nitric oxide. In the present study, we examined the effect of Hemolink™ on the intestinal motor function of anesthetized rats. Treatment of these effects was also studied.

Method: Rats were anesthetized with pentobarbital and the right femoral artery and vein cannulated for blood pressure measurements and infusion of articles, respectively. A saline-filled balloon, made from silicon rubber and attached to a catheter, was introduced into the jejunum. Following a 20 min stabilization period, different doses of Hemolink™, from 0.3 to 1.2 g/kg, were administered. Blood pressure, heart rate and jejunal intraluminal pressure were continuously recorded prior to, during and for up to 40 min after infusion of articles. In a subsequent study, Hemolink™ infusion (0.6 g/kg, the dose which produced the maximal effect) was followed by administration of either morphine, L-arginine, nitroglycerin, glycopyrrolate, or nifedipine to investigate the curative potential of these agents to reverse the Hemolink™ effect on gastrointestinal motility.

Results: Rat whole blood or HSA control articles did not induce any significant hemodynamic or intestinal motor function changes. Hemolink™ administration induced significant intestinal motility changes. Intestinal basal tone, frequency and amplitude of contraction were all increased following the infusion of Hemolink™. The effect appeared to be similar at 0.6 and 1.2 g/kg dose levels, but was less at 0.3 g/kg. The intestinal responses varied considerably among test animals. Hemolink™ administration also caused a mild hypertension and a reciprocal decrease in heart rate. Administration of morphine, L-arginine or glycopyrrolate did not significantly alter the hemodynamic and intestinal mortility changes induced by Hemolink™. These effects, however, were reversed following the administration of either nitroglycerin or nifedipine

Conclusions: Hemolink™ affects the intestinal motor function and systemic hemodynamics which can be treated with either nitroglycerin or nifedipine. This study supports the hypothesis that nitric oxide binding by Hemolink™ plays a role in these effects.

RAPID IN VIVO OXYGENATION OF DEOXY-HEMOLINK™ FOLLOWING 50% OR >90% EXCHANGE TRANSFUSION IN RATS

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Purpose: Hemolink™ in its reduced form (deoxy-Hemolink™) is more stable than oxy-Hemolink™ for storage at ambient temperature. However, for deoxy-Hemolink™ to be useful as an oxygen carrier, it needs to be oxygenated. This can be accomplished either by recharging with air/oxygen prior to administration or following its passes through the lungs in vivo. The objective of this study is to demonstrate the rapid in vivo conversion of deoxy-Hemolink™ to Hemolink™ in a conscious rat exchange transfusion model.

Method: Two levels of exchange transfusion (ET) were performed: 50% and greater than 90%. In 50% ET, the efficacy of deoxy-Hemolink™ as an oxygen carrier was compared with Hemolink™ by measuring the hemodynamic response and blood gases. In >90% ET, the in vivo oxygenation of deoxy-Hemolink™ was followed by CO-Oximetry and compared with Hemolink™. Blood gases and survival (7 days) were also monitored.

Results: In 50% ET, the mean arterial blood pressure increased from 115±3 to 124±3 mmHg in the Hemolink™ group and from 115±4 to 121±2 mmHg in the deoxy-Hemolink™ group (not significantly different). Both groups showed a reciprocal decrease in heart rate corresponding to the pressure rise. PO_2, PCO_2 and pH were not changed significantly following 50% ET in both groups. The results suggested that a rapid oxygenation of deoxy-Hemolink™ occurred in the body and that the product behaved virtually the same as Hemolink™. The rapid oxygenation of deoxy-Hemolink™ was confirmed in the >90% ET study. CO-Oximetry and blood gases were measured immediately after the completion of ET and at 0.5, 1, 2, and 3 hr post ET. Immediately after the completion of ET, the oxy-Hb and reduced Hb were 70% and 20% in the Hemolink™ group and 68% and 25% in the deoxy-Hemolink™ group, respectively. The differences in oxy-Hb and reduced Hb between the two groups after ET and throughout a 3-hour post ET monitoring period were not signifcant. PO_2, PCO_2 and pH measurements for 3 hours following >90% ET were indisguishable for the two groups and all animals from both groups survived the 7-day observation period without apparent problem.

Conclusions: Deoxy-Hemolink™ is rapidly oxygenated to Hemolink™ as it passes through the lungs following in vivo administration. This study also demonstrates that Hemolink™ supports life in the absence of red blood cells in rats.

OXYGEN DIFFUSION MEDIATED WITH MODIFIED HEMOGLOBINS IN AN AQUEOUS MEMBRANE

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We analyzed the oxygen-transporting capability of modified hemoglobins by measuring the oxygen flux across the non-flowing but thin solution membranes of the hemoglobins. The oxygen flux was enhanced with the hemoglobins: e.g. two and more than 10 times for the crosslinked hemoglobin solution over that of the met-hemoglobin control solution at oxygen concentrations of 110 and 20 mmHg at the feed side, respectively. This was in contrast to the simple and physical flux of nitrogen across the hemoglobin solution and of oxygen in the met-hemoglobin solution which responded only to the gas concentration gradient and was independent of the upstream gas concentration.

We ascribed the enhancement in the oxygen flux to the facilitated transport of oxygen or carrier-mediated transport, i.e. the hemoglobin acts as an oxygen-carrying shuttle, picking up oxygen at the feed side, rapidly diffusing across even the non-flowing solution as an oxy form, releasing oxygen to the oxygen-deficient site, and then quickly diffusing back to the feed side in order to repeat the process. The oxygen transport was in the following order: hemoglobin $\sim$ crosslinked hemoglobin $>$ red cells $\sim$ polymerized hemoglobin $\geqq$ PEG-conjugated hemoglobin $>$ liposome-encapsulated hemoglobin.

The transport analysis gave a diffusion coefficient of oxygen that was mediated with hemoglobin and the physical diffusion coefficient of oxygen. The physical diffusion coefficient of oxygen was comparable for the six hemoglobin solutions and was not significantly affected by the solution viscosity. The hemoglobin-mediated oxygen diffusion directly contributed to the enhanced or facilitated oxygen flux and the magnitude of its coefficient was the same as for the above order for the six hemoglobins and approximately coincided each other for the polymerized and conjugated hemoglobins and red cells at a hemoglobin concentration of 10 g/dl. The hemoglobin-mediated diffusion coefficient decreased with the hemoglobin concentration because of an increase in the solution viscosity. This decrease was logarithmical for the acellar modified hemoglobins. The oxygen-delivering profile of the modified hemoglobins was discussed in comparison with red cells.

INTERACTION OF RGD-LIPOSOMES WITH PLATELETS

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The physiology of platelet in hemostasis and thrombosis has been extensively evaluated. Specific membrane glycoproteins and platelet metabolic processes including ca^{2+} mobilization, thromboxane A_2 generation, and granule release, play important roles in platelet function. Recently, liposome-platelet interaction have been focussed on the phagocytosis of liposomes by platelets and the role of platelets in clearing liposomes from the circulation. A family of extracellular matrix adhesion molecules including fibronectin, vitronectin, and fibrinogen, which act on many different cell types, have been demonstrated to contain the adhesive tripeptide arginine-glycine-aspartic acid (RGD). Cell surface receptors of platelets bind to the RGD sequence of small peptides in an activation-independent manner. In this paper, we present the interaction of platelets and liposomes with RGD as a surface ligand (RGD liposomes).

The lipid mixing determined by the resonance energy transfer suggests that the mixing of the lipid of RGD-liposomes with platelets is approximately 4-9 times greater than that for unlabeled liposomes. For the measurement of phagocytosis of RGD liposomes by platelets, fluorescence excitation spectra were measured after incubation of pyranine-liposomes with platelets for various times. Uptake of RGD-liposomes by platelets is 3-4 times greater than that for unlabeled liposomes. For the measurement of changes in the intracellular platelet Ca^{2+} concentration after contact with the liposomes, fluorescence measurements of a Fura-2 loaded platelet suspension, mixed with the liposomes, were made using a Perkin-Elmer Intracellular Biochemistry Application system. The cytoplasmic free Ca^{2+} concentration in platelets is constant at a resting level of approximately 30 nM after contact with RGD liposomes, suggesting that the lipid mixing of RGD liposomes with platelets and uptake of RGD liposomes by platelets are enhanced by the binding of liposome surface RGD ligand to GPIIb-IIIa without causing platelet activation.

These results show the possibility that the liposome-platelet complex can be used as a drug delivery system using a unique ability of platelets to target thrombi, clots, and areas of infection and inflammation.

GENERATION OF SUPEROXIDE FROM HUMAN NEUTROPHIL BY LIPOSOME-ENCAPSULATED HEMOGLOBIN

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Human leukocytes potentially phagocytose liposome-encapsulated hemoglobin (LEH) which is infused as a red cell substitute. Therefore, we have investigated the interaction between NRC (Terumo Inc., Japan), one of LEH, and human polymorphonuclear cells (PMNC) as assessed by superoxide generation. Superoxide was detected by electron spin resonance (ESR) using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap reagent. Either NRC or empty liposome was added to PMNC suspension containing DMPO at a dose of 8% (v/v), and then the kinetics of superoxide generation was examined at room temperature. Empty liposome is identical to NRC, except that it is half in a diameter and dose not contain any proteins.

Both NRC and empty liposome induced the superoxide generation, and this was inhibited by the addition of superoxide dismutase (SOD) to the reaction mixture. The intensity of ESR signal induced by NRC was smaller than that by empty liposome. Because NRC contained SOD copurified with hemoglobin from red cells and its activity still remained, SOD in NRC might partially eliminate superoxide. Interestingly, NRC and empty liposome enhanced superoxide generation induced by phorbol myristate acetate (PMA). In this case, the intensity of ESR signal by NRC plus PMA was also small compared to empty liposome plus PMA.

It becomes clear that NRC induced superoxide generation from neutrophil, but some of superoxide generated was eliminated by SOD of NRC itself. However, the importance of this observation on clinical significance remains to be clarified.

INFLAMMATORY CYTOKINE PRODUCTIONS IN WHOLE BLOOD MODIFIED BY LIPOSOME-ENCAPSULATED HEMOGLOBIN

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Liposome-encapsulated hemoglobin (LEH) has been studied as a red blood cell substitute. Several studies in vivo showed that LEH was trapped by reticuloendothelial systems and subsequently the circulatory levels of cytokines increased. We previously reported that cultured human peripheral blood monocytes phagocytosed LEH and showed several morphological changes. Moreover, the production of several inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor-α (TNF-α) was modified by the coculture with LEH.

To further investigate the effects of LEH on cytokine production, we cultured whole blood with 5% of saline (control), LEH or empty liposome (EL) in a CO_2 incubator and examined the cytokine (IL-6, IL-8 and TNF-α) production. The concentrations of IL-6, IL-8 and TNF-α in control and EL-treated blood slightly increased during 24h incubation. The concentrations of IL-6, IL-8 and TNF-α in blood with LEH increased 100, 30 and 70 fold, respectively, of those in controls 6h after the incubation began. However, 24h after the incubation, cytokine concentrations in LEH-treated blood decreased to the same levels of the controls. In order to mimic the clinical situation with a bacterial infection, we added lipopolysaccharide (LPS) to blood 24h after the addition of saline, EL or LEH and subsequently incubated for 24h. Addition of LPS greatly increased cytokine productions in all groups, and the potentiation was the most prominent in blood with LEH.

In situations when LEH will be used, there will be an underlying inflammatory condition like trauma, infection and so on. There is a possibility that patients suffering from inflammatory conditions will be further given stress attributable to LEH administration. Precise studies are needed.

BLOOD PROGRAM AND THE POSSIBILITY OF APPLYING RED CELL SUBSTITUES IN THAILAND

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Thailand is a country in Southeast Asia, with approximate area of 202,000 sq. miles and total population of 60 millions. Bangkok is the capital city with 6 millions inhibitants.

Thai national blood program is managed by Thai Red Cross Society. The main centre namely National Blood Centre (NBC) is situated in Bangkok. There are 6 branches in Bangkok and 141 branches throughout the country. The NBC operates on a comprehensive blood transfusion service and supply blood components to cover the need of patients in Bangkok and nearby provinces. The branches at each provinces will operate to meet their own provincial demand of blood components.

In 1996, 330,000 units of blood were collected by NBC while total collection of blood from the whole country were 1 million units. All of them were collected from volunteer un-paid donors. Every units were tested for ABO grouping, Rh typing, screening of atypical antibody, screening for syphilis (VDRL), HBsAg (EIA), anti-HCV (EIA), anti HIV (EIA) and HIV Ag (EIA). The HIV Ag testing was introduced since september 1991. It can reduce transfusion associated HIV transmission from 11.4 per million down to 2.5 per million. However, we have to spent US$ 101,594.36 in order to detect one unit of blood which is positive only HIV Ag but not anti HIV. All other efforts and measurements are also time consuming and costly such as education, donor recruitment and retention, predonation coun-selling and donor self exclusion. Blood substitute may be the only answer to solve the problems.

CONSTRUCTION OF NON-ENZYMATIC REDUCTION SYSTEMS OF MetHEMOGLOBIN OF HB-VESICLES

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The hemoglobin vesicle (HbV) has a cellular structure which encapsulates concentrated Hb in the inner aqueous phase of a phospholipid bilayer vesicle. Hb is gradually autoxidized to methemoglobin (metHb), which can not bind oxygen during oxygen transport under physiological conditions.[1]

In order to suppress metHb formation or autoxidation, for the long term maintenance of the oxygen transporting capability, a series of thiols (cysteine, Cys; glutathione, GSH; homocysteine, Hcy; acetylcysteine Acy) were studied as reductants of metHb. Hcy and GSH showed a good suppressive effect on metHb formation, while Cys adversely accelerates the metHb formation in air at a rate twice that of the Hb solution without any reductants, and Acy showed no change.[2] The significant suppression by the coaddition of superoxide dismutase (SOD) and catalase to Cys indicated that Cys was easily oxidized by oxygen and simultaneously generates a large amount of active oxygens. The effective suppression of metHb formation by SOD and catalase was not observed for HbV containing no reductants, indicating that the generation of active oxygens from Hb itself is not significant. The coencapsulation of Hcy with Hb resulted in a low rate of metHb formation in HbV (initial rate: 1%/h) *in vitro* at an oxygen partial pressure (PO_2) of 142 Torr. The rate increased with decreasing PO_2 and showed a maximum (2.2 %/h) at around PO_2 = 23 Torr, and then decreased to 0 %/h at 0 Torr. From these results, it is suggested that the fast metHb formation rate in the blood circulation of Wistar rats injected 20vol% of the HbV solution would be mainly caused by the exposure of HbV to the low PO_2. [2]

On the other hand, we evaluated the reduction of metHb by electron transfer across the bilayer membrane of HbV from a reductant added to the outer aqueous phase. Water-soluble methylene blue (MB) and hydrophobic ubiquinone 10 (UQ) were selected as electron mediators. Under a nitrogen atmosphere, the addition of the reduced form nicotinamide-adenine dinucleotide (NADH) to the outer aqueous phase of UQ-incorporated HbV showed only a slow reduction rate for metHb. On the other hand, when MB and NADH were added under a nitrogen atmosphere to HbV containing 40% metHb, a rapid decrease in the metHb percentage was observed. The entire reaction was controlled by the MB reduction by NADH in the outer aqueous phase. Under aerobic conditions, the decrease in the efficiency of the metHb reduction and rapid oxidation after reaching the minimal metHb percentage were observed. This was confirmed to be due to the influence of hydrogen peroxide; the decrease was prevented by the co-encapsulation of catalase. [3]

In conclusion, the electron transfer through the bilayer membrane of the HbV was possible by the addition of MB with NADH to the HbV. Further investigation relating to MB, such as the toxicity of MB and the retention time of MB efficiency in blood circulation, would be needed.

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The reduction of methemoglobin within Neo Red Cells(NRCs).

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The methemoglobin is reduced by the NADH-Cytochrome b5 reduction system within the Embden-Meyerhof pathway in RBCs. We completely succeeded the reduction of methemoglobin with this system in Stroma Free Hemolysate (SFHL). We evaluated the methemoglobin reduction within the liposome encapsulated hemoglobin (Neo Red Cells: NRCs), but this reduction efficiency was weakened by the encapsulation of SFHL with liposome.

The causes of methemoglobin increasing in NRC were thought that the dissociation of hemoglobin tetramers to dimers, the lack of electron transferred protein in NADH-Cytochrome b5 reduction system and so on.

The dissociation of hemoglobin tetramers to dimers enhances autoxidation of hemoglobin. It was confirmed that the hemoglobin dissociation to dimers were not occurred within the process of NRC production, by using of the native electrophoresis. In addition, the methylene blue as the substitute of electron transfer was studied to enhance the reduction of methemoglobin. The methylene blue was so effective to reduce the methemoglobin formation in SFHL. However, it was not so effective in NRC.

The increase of methemoglobin has relation to the interaction of hemoglobin and lipids. So, the lipids (phosphatidylcholine, cholesterol and myristic acid) swelling process was studied. It was known that the optimum pH of the methemoglobin reduction system was weak alkaline, but swelled lipids was acidic. So the condition of swelling process was improved to the more neutral procedure, then we could achieve the reduction of methemoglobin within the NRCs. It was conformed that the effect of substrates (Glucose, Adenine, and Inosine) and a coenzyme (NAD), and presented methemoglobin reduction.

RHEOLOGICAL PROPERTIES OF SURFACE-MODIFIED Hb-VESICLES

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Hb-vesicles (HbV) which encapsulate a purified and concentrated hemoglobin (Hb) solution with lipid bilayer membranes were studied as oxygen-carrying particles with good rheological properties. When solutions of poly(ethylene glycol) (PEG5000)-conjugated phosphatidylethanolamine or glycolipid conjugating a maltopentaose were added to the HbV suspension, the lipids were spontaneously incorporated into the outer surface of the HbV, and modified the surface with PEG chains or oligosaccharide chains.

The viscosity of the unmodified HbV suspended in saline ([Hb] = 10 g/dL) was 2.6 cP (shear rate = 358 s^{-1}, 37 °C), less than that of human blood (4 cP). However, when suspended in a 5 g/dL albumin solution (HbV/albumin), it increased to 8 cP due to the molecular interaction between albumin and vesicles, and the viscosity increased with decreasing shear rate, e.g., 37 cP at 0.58 s^{-1}. As for the PEG-HbV/albumin, on the other hand, the viscosity was 3.5 cP at 358 s^{-1} and was comparable with that of human blood. Optical microscopy showed formless flocculated aggregates of the unmodified HbV. The aggregates of the Glyco-HbV dissociates reversibly by mild agitation. While no aggregates were confirmed for the PEG-HbV. The steric hindrance of PEG chains seemed to be effective in preventing intervesicular access and the resulting aggregation.

Permeability of HbV through membrane filters having penetrated pores with a regulated size was examined in relation with the degree of aggregation during a capillary flow. Both the unmodified and modified HbV have the higher permeability than blood and lower than stroma-free Hb solution at the same Hb concentration (10g/dL). PEG-HbV and Glyco-HbV showed higher permeability than the unmodified HbV. Thus, the solution properties of the HbV were improved by the surface modification and excellent behaviors in microcirculation would be expected.

The difference in the turbidity of the vesicle dispersion before the addition of polymers and the turbidity saturated after the addition of polymers such as dextran was represented as maxΔO.D. The PEG-HbV, Glyco-HbV, and HbV were used to study the molecular weight dependence of the dextran on the maxΔO.D[1]. The starting point of the maxΔO.D increase with increasing molecular weight is defined as a critical molecular weight (Mc) of polymers for the aggregation of vesicles, and the maxΔO.D increases steeply with the Mc and reach a constant value. It means that the polymers with molecular weight smaller than the Mc have no ability to make the aggregation of vesicles. The Mc of dextran added to the Glyco-HbV is lower than that of dextran added to the unmodified HbV, indicating the interaction between saccharide chain of the glycolipid and dextran. While, the larger Mc of dextran added to the PEG-HbV indicates that the interaction of dextran to the surface of the vesicle effectively suppressed by this pegolation.

Reference:
[1] Bioconjugate Chem. **8**, 23-30 (1997).

OXYGEN DELIVERY STUDIES OF BLOOD AND BLOOD SUBSTITUTES USING OXYGEN-15-LABELED MOLECULAR OXYGEN

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A major problem facing red cell substitute investigators is quantitively assessing the oxygen uptake and tissue delivery of red cell substitutes. Quantitative assessment of this tissue oxygen delivery is important to determine the effect of affinity on oxygen release. We previously developed a technique using the short-lived cyclotron-produced radioistope of oxygen (^{15}O, half-life of 2 minutes) to quantitate the uptake of oxygen in the lungs by the red cell substitute. We have now developed a technique using ^{15}O to measure the release of oxygen from a red cell substitue at the tissue level. This technique uses 2 scintillation probe detectors collimated over the brain of a rabbit. The cyclotron-produced $^{15}O_2$ is bubbled through the red cell substitute and a 0.2 ml bolus of the ^{15}O-labeled-red cell substitute is rapidly injected through the carotid artery of a rabbit while a recording is made of the ^{15}O activity curve. The activity is then decay corrected to account for the rapid decay of oxygen. The curve of activity is biphasic with a rapid phase followed by a slower linear decline in activity. The peak counts represent the total oxygen given while the slow linear washout represents the $^{15}O_2$ that is extracted from the brain and converted to metabolic water. The extraction fraction is determined by back extrapolating the slow linear phase to the injection of the bolus. The short half-life of ^{15}O allows multiple studies of several different red cell substitutes to be repeated at 15 minute intervals in the same rabbit, allowing comparisons of different red cell substitutes with the autologous red cells in the same animal in the same position.

Initial ^{15}O studies have been conducted to determine how long circulating polyethylene-gycol surface modified LEH (PEG-LEH) compares to red blood cells and α–α crosslinked hemoglobin. *In vitro* bubbling studies reveal that PEG-LEH binds a similar quantity of ^{15}O which is comparable with red blood cells and α–α crosslinked hemoglobin containing the same concentration of hemoglobin, while bubbled plasma took up only trace amounts. These studies confirm that the bubbled aliquots have minimal contamination by dissolved oxygen.

In vivo oxygen delivery studies reveal that all three agents studied (red blood cells, α–α cross-linked hemoglobin and PEG-LEH) had extraction fractions of 40-50%, with no significant differences noted between the three oxygen carriers. These initial studies indicate that liposomal encapsulation of hemoglobin and surface modification of LEH with PEG does not interfer with the oxygen carrying properties of the encapsulated hemoglobin.

SURFACE MODIFICATION OF LIPOSOME-ENCAPSULATED HEMOGLOBIN WITH POLYETHYLENE GLYCOL SIGNIFICANTLY INCREASES CIRCULATION PERSISTENCE

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A major obstacle in the development of red cell substitutes has been overcoming their short circulation persistence. Although encapsulation of hemoglobin in a liposome increases its circulation persistence, the circulation half-life of standard formulations of liposome encapsulated hemoglobin (LEH) are still no longer than 20 hours due to removal by the reticuloendothelial system (A. Rudolph, et al. , *PNAS*, 88:10976-10980). Recently, studies have shown that the addition of distearoyl phosphoethanolamine polyethylene glycol (PEG-PE) to liposomes can significantly increase their circulation persistence. Previous attempts to apply this technology to LEH using a formulation containing 5 mol% of PEG-PE containing a headgroup with a molecular weight of 1,900 were unsuccessful in increasing the circulation persistence of LEH (S. Zheng, et al., Art Cells, *Blood Subs, and Immob Biotech*, 22: 487-501). In previous studies of empty liposomes, we have determined that a higher concentration of PEG-PE (10 mol % PEG-PE concentration containing a headgroup with molecular weight of 5000) resulted in the longest liposome circulation persistence in a rabbit model (B. Goins, et al. , *J of Nuclear Med* , 37:1374-1379). The present study was performed to determine if this same PEG formulation would increase the circulation persistence of LEH.

PEG-LEH (containing 10 mol % PEG-5000-PE added at the dried lipid film stage) was radiolabeled with technetium-99m (^{99m}Tc), infused into rabbits (25% of blood pool at 1 ml/min) (n=5) and monitored by scintigraphic imaging at various times out to 48 hours. This PEG-LEH formulation had decreased RES uptake and significantly prolonged circulation persistence in comparison with an earlier formulation of LEH containing 10 mol % dimyristoyl phosphatidylglycerol (DMPG). Although both formulations exhibited biphasic pharmokinetic patterns, the PEG-LEH had a longer elimination phase half-life compared to the previous DMPG formulation (65 hours vs 18 hours). Tissue distribution data at 48 hours revealed that 48.67 ± 3.4 % of the ^{99m}Tc-PEG-LEH remained in circulation, a greater than three-fold increase in the circulation time over DMPG-LEH. The liver had the greatest accumulation at 48 hours (12.69 ± 0.70 % injected dose) followed by bone marrow (6.18 ± 0.14 %), while the spleen had only 1.42 ± 0.2 % of LEH.

This PEG-LEH formulation shows much promise as a second generation long circulating blood substitute.

ADVANCED FLUOROCARBON-BASED SYSTEMS FOR OXYGEN AND DRUG DELIVERY

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The oxygen-delivering fluorocarbon emulsions presently under development differ from the earlier generation of products in multiple respects: the fluorocarbon selected has some lipophilic character and is rapidly excreted; the emulsifier, egg-yolk phospholipid (EYP), has a long history of practice in parenteral lipid emulsions; the emulsions are several-fold more concentrated yet nevertheless fluid; they are considerably more stable; and they do not require any reconstitution or manipulation prior to administration. The most advanced of these products, Oxygent™ (Alliance Pharmaceutical Corp. and Johnson and Johnson), consists in a 60 w/v% concentrated emulsion of a primary fluorocarbon, perfluorooctyl bromide (perflubron), stabilized by a secondary, heavier but lipophilic compound, perfluorodecyl bromide, and emulsified with EYP. It is heat-sterilized, stable for two years at standard refrigeration temperatures, and ready for use. Recent formulation and process optimization led to substantial reduction of the side-effects - a consequence of the natural elimination process of particulates - that were observed with the earlier products; fever has become infrequent and does not exceed 1°C, and platelet counts remain within normal range. Hundred-ton-size manufacture of perflubron is in place as well as a cost-effective, one-million-dose-per-year, emulsion production and sterilization facility. Oxygent has completed Phase II clinical trials for use in conjunction with acute normovolemic hemodilution during surgery. Administration of low doses (e.g. 1.8 g/kg body weight) of the product resulted in immediate and significant increase in mixed venous oxygen tension and correction of physiological indicators for transfusion (hence delays before transfusion of the patient's stored autologous blood is required) thereby reducing the need for allogeneic blood transfusion. Phase II trials for use during cardiopulmonary bypass surgery are in an advanced stage. Novel, room-temperature-stable experimental emulsions have been devised that incorporate fluorocarbon-hydrocarbon diblocks to improve the adherence of the phospholipid coating to the fluorocarbon droplets; these emulsions are being investigated for organ preservation. Further fluorocarbon-based systems are being investigated that have potential in diagnosis and as drug delivery systems. They include fluorocarbon-stabilized microbubbles, suspensions of micro and nano particles in a fluorocarbon, water-in-fluorocarbon reverse emulsions, hydrocarbon-in-fluorocarbon emulsions, multiple emulsions with two or three immiscible phases, and fluorocarbon gels.

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COMPARISON OF OPTRO™ WITH WHOLE BLOOD USING ^{31}P-NMR SPECTROSCOPY

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We have utilized ^{31}P-NMR spectroscopy to determine the efficacy of 5 or 3% recombinant human hemoglobin *(*OPTRO™*)* as an oxygen carrier *in vivo* by monitoring the high-energy phosphorus (HEP) metabolism of the rat abdomen during and after, isovolemic replacement of the blood (to a residual erythrocyte hemoglobin concentration, [Hb]e < 1 g/dl with OPTRO™ and compared with animals similarly exchanged with a 5 % solution of *human serum albumin* (HSA). NMR signals allowed quantitation of creatine phosphate (Pcr), ATP, orthophosphate and tissue pH. In hour-long HSA exchanged rats, significant changes in high energy phosphates (HEP), the sum of PCr and ATP concentration, vs. [Hb]e were observed. For [Hb]e > 10.0, the mean value of [HEP] was 97.5 (± 2.2, n=10)%, while for 2.5 < [Hb]e < 3.5 the mean value of [HEP] was 78.5 (±4.6, n=5)%, a value which is significantly smaller (α=0.0025). At the end of the HSA exchange, [Hb]e < 1, and prior to death, a 4-fold increase in orthophosphate and a 50% decrease in HEP was observed. The tissue pH fell from 7.35 at the start of the experiment to 6.8. In contrast, exchange transfusion with OPTRO™ ([Hb] = 5 g/dl) resulted in no significant decrease in HEP, and no change in orthophosphates or tissue pH. In subsequent experiments, animals were exchange transfused with a 3 % OPTRO™ solution. The results indicate that at [Hb]e > 10.0 g/dl, the mean value of the HEP was 100.0 ± 1.6 % (n=9), while for [Hb]e < 1.0 g/dl the mean value of the HEP was 91.0 ± 3.8 % (n=6). The HEP value determined at a total [Hb] of 3 g/dl with OPTRO™ *(91.*0 %) was found to be significantly higher (p = 0.05) than that found at the same [Hb] with erythrocytes during HSA exchange (78.5 %). These results suggest that OPTRO™ performs better at sustaining HEP than the same hemoglobin content in erythrocytes. The probability that blood and OPTRO™ *(at* [Hb] = 3 g/ dl) perform equally well at supporting the HEP metabolism of the rat gut was determined as a function of [Hb]e. The erythrocyte hemoglobin concentration at which the probability is less than 5 % (p ~ 0.05) that OPTRO™ and blood are the same is 7.03 g/dl, a value that is 2.34 times larger than the actual concentration of hemoglobin in the OPTRO™ solution used. We conclude that OPTRO™ appears to support tissue metabolism at least twice as efficiently as blood in these studies.

EVALUATION OF HUMAN RECOMBINANT HEMOGLOBIN, rHB1.1, IN CELL SALVAGE FOR USE DURING AUTOTRANSFUSION

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Autotransfusion devices used for cell salvage play an integral role in blood conservation strategies within the surgical arena. The ability to utilize this technology with acellular hemoglobin based oxygen carriers, HBOCs, should further reduce the requirements for allogeneic transfusion. However, cell salvage devices incorporate an optical sensor to determine, following continuous centrifugation, the plasma-erythrocyte interface. Therefore it was necessary to determine whether rHb1.1 would cause the optical sensor to trip prematurely to initiate a wash cycle in the automatic mode and furthermore at what concentration of plasma rHb1.1 could cell salvage devices be used in the automatic versus manual mode. In the present study rHb1.1 was mixed with bovine blood, initial hematocrit 36%, at clinically relevant concentrations to achieve plasma free hemoglobin levels between 0.7 to 2.5 gm/dl. Cell salvage was performed using a Medtronic Sequestra 1000^{TM} with a calibrated flow rate of 300 ml/minute and a centrifugespeed of 5600 RPM. The Sequesta 1000 was placed in the automatic mode and the bowl was allowed to fill until the level sensor switched to the wash cycle. If the bowl was not filled to within 2.5 mm of the designated level prior to shifting to WASH then the test failed due to premature tripping of the optical sensor. The results of these studies demonstrated that the one unit (25 gm) dose of rHb1.1 did not result in premature termination of the FILL cycle. The two unit dose resulting in a plasma hemoglobin concentration between 1.3 to 1.7 gm/dl yielded variable results. At a plasma hemoglobin concentration of 1.3 gm/dl, the Sequestra 1000 completed the cycle in the automatic mode while in a separate run, a plasma concentration of 1.7 gm/dl resulted in premature tripping of the sensor. All subsequent runs in which the plasma hemoglobin concentration was greater than 1.7 gm/dl consistently resulted in a premature shift to the WASH cycle. However, the separation between the packed red cell layer and the hemoglobin containing plasma was clearly visible and accentuated by the leukocyte layer at all concentrations of rHb1.1. These results suggest that the manual method of level detection will perform well when rHb1.1 plasma concentrations are anticipated to exceed 1.4 gm/dl.

INFLUENCE OF rHB1.1 RECOMBINANT HEMOGLOBIN ON BLOOD RHEOLOGY

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The ability of acellular hemoglobin based oxygen carriers to decrease blood viscosity and also deliver oxygen throughout the vasculature represents a potential advantage of this class of therapeutics. In the present study, the effects of varying concentrations of rHb1.1 on the rheology of human blood was determined in vitro. Recombinant hemoglobin as a 5% solution had a viscosity of 0.80 mPa.s at 37 degrees C which was lower than autologous hemoglobin solutions at the same concentration (0.93 mPa.s $p < 0.001$) or when compared to albumin (0.93 , $p < 0.001$). The mixture of rHb1.1 to plasma or to erythrocyte suspensions resulted in a dose dependent decrease in their viscosity. rHb1.1 in addition did not affect erythrocyte aggregation or the deformability of erythrocytes or neutrophils as measured by cell transit time through 5 and 8 micron pores. These results suggest that rHb1.1 has excellent rheological properties and will provide additional benefit by decreasing blood viscosity, without activating leukocytes, while providing oxygen carrying capacity.

PHYSIOLOGIC EFFECTS OF HUMAN RECOMBINANT HEMOGLOBIN, rHB1.1 DURING CARDIOPULMONARY BYPASS IN AN OVINE MODEL

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The ability to incorporate acellular hemoglobin based oxygen carriers, HBOCs, into cardiopulmonary bypass, CPB, protocols would permit sequestration of the patients' blood which would avoid the sequella associated with exposure of whole blood to the extracorporeal circuit. However, the effects of CPB on HBOC stability and the effects of HBOCs on surgical and post-surgical hemodynamics require further understanding in large animal models. In the present study, 900 ml of native blood were removed from sheep and replaced with an oxygen carrying equivalent of rHb1.1 or with a similar quantity of autologous red cell hemoglobin plus 5% human serum albumin as colloid. Under these conditions the rHb1.1 treated sheep had a concentration of acellular HBOC comparable with a 4 unit dose in man. Each experimental group consisted of 4 colloid and 4 rHb1.1 treated sheep. CPB was performed in the first phase of the study under normothermia without aortic cross-clamp, in the second phase during hypothermia without ischemia and finally with hypothermia and cardioplegiac arrest. Hemodynamic and oxygenation parameters were evaluated during 90 minutes of CPB and for an additional 60 minutes after removal from the pump. The results of these studies demonstrated that using membrane oxygenators, there was no significant increase in methemoglobin nor did rHb1.1 have any detectable effect on the oxygenator or filters. In all three experimental conditions, all animals were capable of supporting their own circulation following separation from the pump. Furthermore, there were no significant differences in the hemodynamic parameters, specifically in the vascular resistances of the systemic or pulmonary circulation. Oxygenation as PaO2 did not differ significantly between the rHb and control animals although the oxygen content, based on the total hemoglobin concentration differences, did. Therefore, blood replacement with rHb1.1 was well tolerated in this ovine model of CPB and there were no treatment related increases in cardiac dysfunction or vasoconstriction.

RECOMBINANT HEMOGLOBIN, rHB1.1, ENDOTOXIN INTERACTIONS IN VIVO: EFFECTS ON SYSTEMIC TNF AND IL-6 LEVELS IN LETHAL AND SUBLETHAL MURINE MODELS OF ENDOTOXEMIA

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The effects of acellular hemoglobin based oxygen carriers in preclinical models of sepsis and endotoxemia have been inconclusive with regards to outcomes reported for survival. In the present study, mice were infused with 1 gm/kg of recombinant human hemoglobin, rHb1.1, and the effects on mortality and systemic TNF and IL-6 levels were determined using both lethal and sub-lethal bolus endotoxin challenge. Pretreatment of mice with rHb1.1 and challenged with 20 mg/kg of LPS, LD_{100}, resulted in 100 percent mortality by 20 hrs while the same mortality with the vehicle or 5% albumin groups occurred at 50 hrs. Mice challenged with lower LPS concentrations of 10 and 2.5 mg/kg, corresponding to an LD_{15} and an LD_0, respectively, had 100 % and 17% mortality in the rHb group and 17 % and 0 % mortality in the vehicle treated animals. These doses of LPS resulted in maximal increases in systemic TNF and there were only modest differences between the rHb and the vehicle groups at LPS challenge doses of 2.5 and 20 mg/kg while no difference was observed at the 10 mg/kg concentration. At LPS concentrations below 10 ug/kg, the increases in circulating TNF were dose dependent and no differences were observed in serum TNF between the rHb1.1 and vehicle groups. In addition, there were generally no differences in IL-6 between the experimental groups although at 10 mg/kg LPS, a two fold increase in plasma IL-6 levels over the controls was observed in the rHb1.1 treated animals. Infusion of rHb1.1 alone did not induce any increase in circulating IL-6 or TNF. These data demonstrate that although endotoxin exacerbation was apparent at the highest doses of LPS, at clinically relevant concentrations there were no differences in the extent of cytokine elevation nor on survival when comparing rHb1.1, albumin, or vehicle pretreated animals.

RECOMBINANT HUMAN HEMOGLOBIN, rHB1.1 DOES NOT POTENTIATE LOW-GRADE GRAM NEGATIVE BACTERIAL INFECTION IN RATS

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The effects of acellular hemoglobin based oxygen carriers, HBOCs, on bacterial infections remains controversial with HBOCs reported to have no effect, to improve and also to worsen outcome in various preclinical models. The present study was designed to evaluate the effects of recombinant hemoglobin, rHb1.1 on survival when animals were provided with a non-lethal bacterial challenge 18 hrs prior to an exchange transfusion with either rHb1.1 (n=11), a comparable concentration of human serum albumin, HSA (n = 16) or autologous blood (n = 9). Sprague-Dawley rats under anesthesia were inoculated peritoneally with a gel capsule containing 5 x 10^3 CFU of E. coli (strain Sm 018) combined with 35 mg of sterile rat feces. This dose of bacteria coupled with a 30% blood volume exchange transfusion resulted in a 7 day mortality of 0-10% while an inoculum of 1.2 x 10^4 organisms resulted in a 90% mortality. All rats received ampicillin, 85 mg/kg, at 6 hrs post pellet implantation and twice per day for the next two days. Blood samples at 6, 18, 30, and 56 hrs were obtained for CFU and leukocyte counts. Animals that survived at day 7 were considered long term survivors. The results of these experiments demonstrated that mortality between these three groups was between 10-20% and was not statistically different ($p = 0.26$). There were no significant differences in CFU or leukocyte counts. In contrast, an additional group of animals that were administered a non-specific nitric oxide synthase inhibitor, L-NAME, at 25 mg/kg, exhibited a 60 % mortality. Therefore, the use of rHb1.1 at a concentration of 1 gm/kg did not result in exacerbation of an occult infection when used in a clinically relevant protocol.

SUBCUTANEOUS MICROVASCULAR RESPONSES TO HEMODILUTION WITH HB-VESICLES (HbV) [1]: EFFECT OF PEG-MODIFICATION.

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Phospholipid vesicles encapsulating purified Hb (Hb vesicles (HbV); diameter, 258 ± 57nm; P_{50}, 31 mmHg; [Hb], 5 and 10 g/dL) have been developed to provide O_2 carrying capacity to plasma expanders. Their function as a blood replacement was tested in the subcutaneous microvasculature in conscious Syrian Golden hamsters during severe hemodilution where 80% of the red blood cell mass was substituted with suspensions of vesicles in 5% human serum albumin (HSA) solution.

Vesicle membranes were unmodified (HbV/HSA) or conjugated with polyethyleneglycol (PEG) on the surface (PEG-HbV/HSA). The viscosity of 10 g/dL HbV/HSA is 8 cP at 358 s^{-1} due to the intervesicular aggregation, while that of 10 g/dL PEG-HbV/HSA is 3.5 cP since PEG chains inhibit aggregation [1]. Both materials yielded normal MAP, HR, and blood gas parameters at all levels of exchange which could not be achieved with HSA alone. Subcutaneous microvascular studies showed that PEG-HbV/HSA significantly improved microhemodynamic conditions (blood flow rate, functional capillary density, vascular diameter, pO_2) relative to unmodified HbV/HSA. Even though the enhancement of PEG modification did not achieve the functional characteristics of the blood perfused microcirculation, PEG reduced vesicular aggregation and viscosity improving microvascular perfusion relative to the unmodified type.

These results highlight the significance of microvascular analysis in the design of red cell substitutes and the necessity of surface modification of HbV to prevent aggregation.

[1] Sakai, et al. *Bioconjugate Chem.* 7, 24 (1997)

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SUBCUTANEOUS MICROVASCULAR RESPONSES TO HEMODILUTION WITH Hb-VESICLES (HbV) [2]: WHAT IS THE OPTIMAL O_2 AFFINITY (P_{50})?

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One advantage of HbV is that oxygen affinity (P_{50}) is controllable by the amount of coencapsulated allosteric effectors. It has been suggested that higher oxygen availability from acellular type red cell substitutes may cause microvascular hypoxia following vasoconstriction due to autoregulatory response. This study was aimed at determining the relationship between the P_{50} and microvascular perfusion when PEG-HbVs with various P_{50} are administered to conscious Syrian golden hamsters with dorsal skinfold windows.

Three kinds of PEG-HbVs suspended in 8% HSA are prepared with different P_{50}: 9, 16, and 30 mmHg, by coencapsulating pyridoxal 5'-phosphate (PLP) at the molar ratios of [PLP]/[Hb] = 0, 1, and 3, respectively (*cf.* hamster blood, P_{50}: 28 mmHg). Other physicochemical parameters of the three PEG-HbVs are almost identical, *e.g.* particle size (250 nm), [Hb] (10 g/dl), viscosity (4 cP, at 230 s^{-1}).

During the 80% blood substitution with the suspensions, all three PEG-HbV groups showed stable MAP and HR which can not be sustained with HSA alone. Only the 9 mmHg group showed an increase in PaO_2, indicating hyperventilation. Microvascular perfusion and oxygen tensions of the PEG-HbV groups were much higher than in the 8% HSA group. These parameters were further improved by increasing oxygen affinity from 30 mmHg to 16 mmHg, which appears to be an optimal value for the P_{50} in their system. Further increasing P_{50} to 9 mmHg reversed the trend.

This result indicates that the optimal oxygen dissociation curve of PEG-HbV in normoxic condition may be left shifted.

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SUBCUTANEOUS MICROVASCULAR RESPONSES TO HEMODILUTION WITH Hb-VESICLES (HbV) [3]: ACELLULAR Hb vs. ENCAPSULATED Hb.

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α-α Crosslinked Hb (DCLHb) is one of the most widely tested acellular blood substitutes. Therefore, we aimed to compare the microvascular responses to the infusion of an encapsulated type, PEG-HbV, and DCLHb using conscious Syrian golden hamsters fitted with dorsal skinfold windows.

DCLHb was synthesized according to the literature (P_{50}, 30 mmHg; [Hb], 7 g/dl, pH 7.4) [1]. The completion of crosslinking was confirmed with SDS-PAGE electrophoresis. PEG-HbV was regulated to the same P_{50} of 30 mmHg, and suspended in 8% HSA to control the oncotic pressure close to 8% DCLHb. During 80% blood substitution, the DCLHb group showed vasoconstriction and significant reduction in blood flow, and functional capillary density. Hemodilution with PEG-HbV showed higher microvascular perfusion, and there was no significant vasoconstriction. Abdominal incision was made 2 hours post the substitution, and the DCLHb group showed intraperitoneal bleeding, while there was no bleeding for the PEG-HbV group.

These results demonstrates that acellular Hb is vasoactive inducing vasoconstriction and extravasation. These effects were not seen with PEG-HbV, suggesting that encapsulation of Hb in a RBC-like structure is advantageous.

[1] Chatterjee et al., *J. Biol. Chem.* **26**, 9929 (1986).

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FLUOROCARBONS IN OPEN HEART SURGERY

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Cardiopulmonary bypass (CPB) is known to be accompanied by significant blood constituents damage. The use of unemulgated fluorocarbons allows to decrease this negative effect. It results in the necessity to investigate the hemoprotective and other effects of perfluorodekaline (PFD).

Experimental observations in vitro on fluorocarbon processing of preserved donor blood have shown, that PFD decreases an erythrocyte trauma during subsequent contact oxygenetion. Further, for unemulgated fluorocarbon usage in clinical practice the original device for hemoprotection has been designed and evaluated in experiments on animals. As a result, an optimal regime of this device operations has been chosen, as well as blood and PFD hemohydrodynamical interactions have been estimated. This hemoprotective device being integrated into extracorporeal circuit has made it possible to optimaze blood processing with speed 180-200 ml/min.

The designed device for hemoprotection on a basis of PFD using has been applicated in open heart surgery with CPB and buble oxygenation in 14 patients (men from 46 to 68 years old). The partial processing of blood by PFD under CPB conditions increases acid resistance of erythrocytes, modifies its reological properties (deforming ability and viscosity). Free hemoglobin level after 120 min of CPB appeared to be 0,328±0,018 g/l, whereas this parameter in control group (without fluorocarbon processing of blood, n=21) after the first 60 min of CPB has already accounted for 0,285±0,19 g/l and after 120 min - 0,576±0,045 g/l. The electron microscopy scanning of blood samples has confirmed insignificant morphological changes of erythrocytes during CPB with fluorocarbon processing of blood.

Stabilization of red blood cells structural and functional status by extracorporeal fluorocarbon processing of the blood has allowed to reduce the blood transfusion in early postoperative period by 30-35 %, that proves the designed device efficacy.

USE OF PERFTORAN IN EXPERIMENTAL APNEA MODEL

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Gas exchange capabilities of perftoran have been studied in experimental apnea model in 22 mongrel dogs using inferior cava vein - external jugular vein circuit with membrane blood oxygenation for life-term prolongation in acute extreme hypoxia. At sufficiently low flow rate of such perfusion (35 ml/kg×min) and under emergency circumstances, fluorocarbon emulsion (concentration 40 ml/kg) as hemodilutant does not significantly improve oxygen delivery to body in comparison with traditional plasma substitutes. However, perfusion with oxygenated perftoran in membrane oxygenator allows to increase the life-term of animal (50,7±4,3 min), than that with polyglukin (26,0±2,7 min) because the perftoran supports optimal functioning of heart and brain and improves microcirculation. Experimental evidence have been obtained, that central hemodynamics and cardiac rythm disorders against extreme hypoxia background appeared to be much less expressed just in perftoran recipients group. The use of perftoran as a component of cardioplegic solution seems to be perspective for more effective cardiac protection against myocardial ischemia and reperfusion injury during open heart surgery with cardiopulmonary bypass.

CLINICAL STUDIES OF HEMASSIST™

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HemAssist™ (a 10% solution of diaspirin cross-linked hemoglobin) in lactated electrolyte is a modified human hemoglobin therapeutic that has been under clinical evaluation since 1992. Twelve separate patient studies have been completed and three Phase III trials are in progress. The early Phase II trials evaluated the pharmacodynamic effects of this new product in surgical, stroke, intensive care, and hemodialysis patient populations. Later protocols used larger doses in cardiac, orthopedic and general surgery. (A pivotal efficacy trial in 200 cardiac surgery patients was recently completed.)

The results from these studies will be summarized and a status report on the overall drug development process will be given.

THE IMPACT OF BLOOD SUBSTITUTES ON THE BLOOD PROGRAM

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Transfusion safety and self-sufficiency through non-remunerated voluntary donation are the two major tasks of national blood program both for developing and developed countries.

Through the development of the highly sophisticated laboratory screening tests and the virus inactivation techniques, blood transfusion has become quite safer, but still there are risks of adverse reactions, and we haven't reached the "*zero*" adverse reactions yet.

To avoid such reactions, the strategies for reducing allogeneic blood transfusion, and increasing the use of non-human derived artificial blood substitutes and/or performing the substitute therapy, which doesn't rely on human-derived blood, have been promoting.

On the other hand, the tragedy of HIV-infection in hemophiliacs because of the imported coagulation factors had prompted us to be self-sufficient in not only blood for transfusion but also source plasma for plasma derivatives by voluntary donated blood. We already achieved the self-sufficient in blood for transfusion but that of source plasma for fractionation has remained as a serious problem in Japan. To be self-sufficient, new donation methods, 400ml and apheresis donations have been introduced in addition to the conventional 200ml donation in 1986. The rapid increase in the number of donations by the new donation methods enabled us to supply enough coagulation factors derived from voluntary donated blood in 1991 as scheduled, however we are still far from the goal of complete self-sufficiency as a whole. Currently, the recombinant F VIII products are used clinically in Japan, occupying 35 to 40 % of all the demand. The over-consumption of albumin has not resolved yet, and the development and introduction of the recombinant albumin is desired.

Under such circumstances, it can be said that the artificial blood is highly desired for both blood safety as well as self-sufficiency in blood products.

In my speech, I would like to overview the current status of blood program in Japan, and speak focusing on the impact of blood substitutes on the blood program and the perspectives of the application of them.

HUMAN MODIFIED HEMOGLOBIN - BASED BLOOD SUBSTITUTE

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Stroma-free human hemoglobin was pyridoxylated (P-PHb). Its medico-biologic and physico-chemical parameters are the following: P_{50} 26-28 torr at 37^0 C and pCO_2 40 torr in 0.05 M Tris-buffer, pH 7.4; Bohr effect 0.4, MW 120 kD,
MetHb 3%, half-life 12-18 hours. P-PHb can be stored in lyophilized and stabilized form for 2 years. The blood substitute has hemodynamic and gas-transport capability. Preclinical studies of the solution in experiments with different species (mice, white rats, guinea pigs, rabbits, dogs) have shown that the solution has no toxis effects on the central and peripheral organs of immunity. In extensive blood loss, hemodynamic effects of P-PHb is comparable to those of Dextran solutions. Differences between them consist in that immediately after P-PHb infusion the arterial pressure and total peripheral vascular resistance are, as a rule, higher than after Dextran. Hemoglobin levels in blood after P-PHb infusions, due to the Hb presence in plasma, decrease to a less extent as compared to that would be anticipated taking into consideration the hemodilution extent (according to hematocrit). Blood oxygen capacity after infusion of 1% P-PHb solution appears to be higher than after administration of the same amounts of Dextran solutions (control experiments). On that ground, a total amount of oxygen supplied to tissues (the systemic transport of oxygen) is by some 8-10% higher than in the control. Contribution to the oxygen supply to tissues and organs, of P-PHb administered to the vascular bed as a constituent of Hb solution, in dose of 25-30 ml/kg of body weight, has been confirmed by direct measurements of its oxygenation in plasma of arterial blood and deoxygenation in plasma of venous blood.

A NEW APPROACH ON THE TRANSFUSIONAL BLOOD RESEARCH IN TAIWAN, R.O.C.

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Our research are recently focused on two fundamental issues concerning the antigenesity and the temperature stress of hemoglobin based oxygen carriers. Although hemoglobin has been concern as a relatively low antigenic molecule and its epitopes have not been characterized. By protein engineering and immuno-analyses of native and recombinant hemoglobins, we attempt to identify the structural domains of human hemoglobin epitope and the temperature stress of O_2 transport.

Our preliminary results indicate that the temperature stress of Hb function is allosteric regulated and the antigensities of hemoglobins in some domestic animals, such as pig, goat, rabbit and cow are heterogenerous to that of human HbA.

APPLICATION OF PERFLUOROCARBON EMULSIONS TO PROFILACTICE AND TREATMENT OF ACUTE INTOXICATIONS

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The present ivestigations are based on the data concerning the ability of perfluorocarbons to modify biochemical detoxication systems, to tranfer oxygen, and to improve the rheological properties of blood.

The effects of perfluorocarbon emulsion Perftoran (Pushchino, Russia), employed both profilactically and therapeutically, on the resistency of experimental animals towards organophosphorus pesticides, galogen alcans, GABA-blocking agents, MetHb-forming agents were studied. The effectivity of Perftoran in treatment of carbon monooxide, malathion, dichloroethane and drug intoxications was evaluated under clinical conditions.

The effects of profilactical and therapeutical usings of Perftoran were different. It depended on the velocity and the ways of metabolic tranformation of poisons. Perftoran under certain conditions increased the antidotal activity of atropine, dipiroxime, acetilcysteine and others.

The effectivity of Perftoran was revealed at some extracorporal detoxication manipulations such as blood exchange operation, hemosorbtion, hemodialysis.

Perftoran demonstrated therapeutical activity at the treatment of exotoxic shock.

The obtained experimental and clinical data are taken in to consideration in the development of indications for the application of perfluorocarbon plasma substitutes in toxicological practice.

EVALUATION OF POLYETHYLENE GLYCOL-CONJUGATED BOVINE HEMOGLOBIN IN HEALTHY HUMAN VOLUNTEERS

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Polyethylene glycol (PEG) conjugation to proteins has been shown to prolong vascular retention times and to blunt immune or allergic reactions. PEG-L-asparaginase and PEG-adenosine deaminase have been FDA approved for acute lymphoblastic leukemia and adenosine deaminase deficiency associated with severe combined immunodeficiency, respectively.

That many solid tumors are hypoxic has been known since the 1950's. That increasing tumor oxygenation will result in sensitization to radiotherapy and chemotherapy has also been proposed. Recently, we have shown in tumor implant models in rodents that PEG bovine hemoglobin can reverse tumor hypoxia and sensitize tumors to radiation therapy. Similar results have been observed in dogs presenting with spontaneous nasal carcinoma.
Studies in tumor bearing rodents have also confirmed the ability of PEG bovine hemoglobin to sensitize tumors to a wide range of chemotherapeutic agents.

PEG bovine hemoglobin has completed a Phase Ia escalating dose safety evaluation in healthy volunteers. A Phase Ib escalating multiple dose trial in cancer patients receiving radiotherapy is ongoing. A summary of results will be presented.

A NOVEL FREE-HEMOGLOBIN BASED BLOOD SUBSTITUTE: FURTHER EVALUATION OF ITS MOLECULAR EFFECTS ON HUMAN MICROVASCULAR ENDOTHELIAL CELLS

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It has been reported that hemoglobin (Hb) can up-regulate secretory activities of the reticuloendothelial cell system and increase the expression of pro-inflammatory mediators. Furthermore, Hb has been shown to generate and react with both reactive oxygen and lipid species, producing damage to plasma membranes. In our recent study [*Artif Cells Blood Substit Immobil Biotechnol 25(1&2):193-210,1997*] we provided new evidence that certain Hb solutions can activate the transcriptional factor, NF-kappa B, and that its activation can be induced by Hb-mediated injurious oxidative stress. Since the oxidative stress-sensitive NF-kappa B is essential for the inducible expression of the specific set of genes involved in inflammation, these results suggest that its activation can be considered as a "bridge" between Hb-induced oxidative stress and Hb-mediated inflammatory responses. We also showed that chemical modification techniques used in the preparation of free Hb-based blood substitutes may attenuate these pro-inflammatory and oxidative responses, which indicates that development of a non-toxic Hb solution is possible. This study, however; did not focus on a detailed mechanism of the Hb's action. Therefore, using a model of human coronary artery endothelial cells (EC) in culture, in the present study we further investigated their molecular responses to unmodified bovine Hb (U-Hb) and modified Hb solutions which were: (1) polymerized with glutaraldehyde (GLUT-Hb), and (2) prepared according to the patented procedure, which comprises Hb cross-linked intramolecularly with *o*-ATP and intermolecularly with *o*-adenosine, and which is combined with GSH (Hb-PP-GSH, *U.S. Patent No. 5,439,882*). In this study, the EC responses to different Hb solutions were investigated by analysis of the intracellular ionized calcium concentration $[Ca^{2+}]_i$ and intracellular pH by using ratiometric measurements with fluorescence indicators FURA-2 and SNARF-1, respectively. Additionally, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reduced glutathione (GSH) which play critical roles in protection of vascular endothelium from oxidative injury, were determined intracellularly by using spectrophotometrical methods. Results indicate that while U-Hb can be classified as an active molecule able to stimulate EC, the modified Hb solutions introduced different effects. Incubation of EC with U-Hb resulted in a higher intracellular Ca^{2+} influx and a significant drop in the pH ratio, as compared to the control of unstimulated cells. The GLUT-Hb was found to promote even larger accumulation of $[Ca^{2+}]_i$ and a significant intracellular acidification. In this study Hb-PP-GSH did not appear to increase intracellular Ca^{2+} influx nor change the intracellular pH. The degree of oxidative stress to EC was shown to be Hb modification type dependent. We found that the largest depletion of GSH was associated with GLUT-Hb, while the Hb-PP-GSH did not produce any significant changes, and U-Hb generated moderate oxidative stress. While all Hb solutions altered the cellular enzymatic anti-oxidative defense system, a higher SOD/CAT index was observed following the stimulation of EC with GLUT-Hb and to a lesser extent with U-Hb. In conclusion, the results of this study revealed that the higher endothelial calcium influx was correlated with the magnitude of Hb-mediated oxidative stress. The observed rapid perturbation in $[Ca^{2+}]_i$ and intracellular pH may be responsible for the activation of proteases and/or nucleases which may promote the earlier reported endothelial nuclear responses. In this study only Hb-PP-GSH appeared to be non-toxic to EC. This effect can be linked with the anti-inflammatory, homeostatic and cytoprotective properties of adenosine which was used in our preparation as an intermolecular cross-linker and surface modifier, and seems to be the type of chemical modification procedure which lowers the Hb pro-oxidant potential. Thus, appropriate chemical & pharmacological modification of the Hb molecule is required in order to eliminate pro-inflammatory nuclear responses of the endothelium.

COMPARISON OF RESUSCITATION WITH DIASPIRIN CROSSLINKED HEMOGLOBIN VS FRESH BLOOD IN A RAT BURN SHOCK MODEL.

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Diaspirin crosslinked hemoglobin (DCLHb™; Baxter Healthcare Corp) is a vasoactive oxygen-carrying blood substitute which, in our laboratory, improved hemodynamic parameters in a rat burn shock model. Our objective was to compare the effects on hemodynamic parameters and metabolic acidosis of resuscitation with different doses of fresh blood (FB) vs DCLHb.

Methods: Male Wistar rats (200 to 250g), surgically prepared for an acute study, were randomly assigned to one of five treatment groups.(n=8).

I. SHAM (not burned, not resuscitated)
II. DCLHb 2 cc/kg/% Total Body Surface Area (TBSA) burn and 2 cc/kg/% TBSA burn of Lactated Ringers (LR)
III. DCLHb 1 cc/kg/% TBSA burn and 1 cc/kg/% TBSA burn of LR
IV. FB 2 cc/kg/% TBSA burn and 2 cc/kg/% TBSA burn of LR
V. FB 1 cc/kg/% TBSA burn and 1 cc/kg/% TBSA burn of LR

After placement of indwelling catheters, baseline hemodynamic values (mean arterial pressure (MAP), cardiac output (CO), stroke volume (SV), systemic vascular resistance (SVR) and base excess (BE)) were obtained. The animals were immediately intravenously resuscitated after receiving a 30% scald burn and were followed for 6 hours. Resuscitation was based on the Parkland formula (4 X %TBSA burn X animal weight in Kgs = fluid resuscitation for the first 24 hours). This predicted amount is given intravenously, half of the amount is given during the first eight hours postburn and the rest is administered in the remaining 16 hours. The standard fluid used is Lactated Ringers. Blood was obtained from donor male Wistar rats. The animals were euthanized at 6 hours. Groups were compared by one way ANOVA.

Results: MAP remained within normal range in all groups. The SVR, CO, SV and BE were normalized earlier in the LR-DCLHb groups when compared to the LR-FB groups ($p<0.05$). SVR was normalized by 3 hours in group II vs 4 hours in group III ($p<0.05$). CO and SV were normalized by 1 hour in group II vs 4 hours in group III ($p<0.05$). However BE was normalized by 1 hour in group III vs 6 hours in group II ($p<0.05$).

Conclusions: Early resuscitation with DCLHb is superior to FB in improving hemodynamics in this model. There appears to be a direct relationship between dose an effect with the use of DCLHb. DCLHb could be useful in decreasing resuscitation fluids in acute burns without compromising general tissue perfusion.

DIASPIRIN CROSSLINKED HEMOGLOBIN (DCLHb™) IMPROVES HEMODYNAMIC PARAMETERS, BASE DEFICIT, AND SURVIVAL IN A RAT BURN SHOCK MODEL.

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Diaspirin crosslinked hemoglobin (DCLHb™; Baxter Healthcare Corp) is a vasoactive oxygen-carrying blood substitute which has been shown to improve base deficit in numerous experimental studies of shock. Our objective was to determine if adding DCLHb to the resuscitation regimen would improve hemodynamic parameters, metabolic acidosis and survival in our rat burn shock model.

Methods: Male Wistar rats (200 to 250g), surgically prepared for an acute study, were randomly assigned to one of three treatment groups, all with n=8:

I. Lactated Ringer's (LR) 4 cc/kg/% Total Body Surface Area (TBSA) burn
II. LR 2 cc/kg/% TBSA burn and 2 cc/kg/% TBSA burn of human serum albumin (HSA)
III. LR 2 cc/kg/% TBSA burn and 2 cc/kg/% TBSA burn of DCLHb

After placement of indwelling catheters, baseline hemodynamic values (mean arterial pressure, cardiac output, stroke volume and base excess) were obtained. Rats were immediately intravenously resuscitated after receiving a 30% scald burn and were followed for 6 hours. Resuscitation was based on the Parkland formula (4 X %TBSA burn X animal weight in Kgs = fluid resuscitation for the first 24 hours). This predicted amount is given intravenously, half of the amount is given during the first eight hours postburn and the rest is administered in the remaining 16 hours. The standard fluid used is Lactated Ringers. Over the 6 hours animals received a total amount of intravenous fluid between 9.00 cc to 11.25 cc depending on their weight. Animals surviving 6 hours were euthanized. Groups were compared by one-way ANOVA.

Results: Mean arterial pressure, cardiac output, stroke volume and base excess were all improved in DCLHb-LR treated animals compared to other treatment groups at 3, 4, 5 and 6 hours postburn ($p < 0.05$). The 6-hour mortality rates were 0/8 (LR-DCLHb group); 3/8 (LR-HSA group) and 6/8 (LR only group).

Conclusions: Early resuscitation with DCLHb is superior to non-O_2-carrying resuscitation fluids in improving hemodynamics and survival in this model of burn shock. DCLHb may improve general tissue perfusion in the acute postburn period and could be useful in early management of patients with severe burns.

HEME OXYGENASE IN RAT LIVER: TOPOGRAPHIC BASIS AND MECHANISMS FOR CARBON MONOXIDE-MEDIATED MICROVASCULAR RELAXATION

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We have previously showed that carbon monoxide (CO) generated by heme oxygenase (HO) plays a modulatory role in controlling microvascular tone in the liver (*J. Clin. Invest. 96, 1995*). However, intrahepatic distribution of this CO-generating enzyme remains unknown. This study aimed to examine distribution of the HO isoforms in and around hepatic sinusoids. According to immunohistochemistry using monoclonal antibodies against HO-1 and HO-2, the inducible and constitutive isoforms of HO, the liver expressed both isoforms with distinct topographic patterns: HO-2 distributed in parenchymal cells, while HO-1 was expressed abundantly in Kupffer cells. Both isoforms were undetectable in hepatic stellate cells. Hepatocytes seemed to constitute a major cellular component for endogenous CO generation; freshly isolated hepatocytes were able to generate CO at a rate of 1 nmol / min / g liver, which is almost stoichiometric to that measured in the venous effluent from the perfused liver. In the perfused liver, administration of HbO_2, a CO-trapping reagent that could access the space of Disse, elicited a marked sinusoidal constriction, whereas liposome-encapsulated Hb which was unable to enter the space did not induce such a vascular response. These results indicate that CO generation in the extrasinusoidal space is indispensable for physiological relaxation of hepatic sinusoids and thus suggest requirement of liposomal encapsulation to develop blood substitutes that can disribute specifically in the intravascular space in organs possessing fenestrated endothelium.

LIPOSOME ENCAPSULATION OF HEMOGLOBIN: A STRATEGY FOR BLOOD SUBSTITUTES TO GUARANTEE PHYSIOLOGICAL HEPATOBILIARY FUNCTION

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Modified hemoglobin derivatives are going to be applied clinically as artificial blood substitutes, although their side effects on organ functions are not fully examined. This study aimed to demonstrate that liposome encapsulation of hemoglobin is necessary to maintain physiological organ functions particularly in liver, a major organ constituting reticuloendothelial system that allows free hemoglobin to access extravascular space and to thereby scavenge vasorelaxing gaseous monoxides such as carbon monoxide. In rat perfused liver preparation, administration of diaspirin-crosslinked hemoglobin (XLHb, 1.5 g/dl in Hb concentration) evoked a significant increase in microvascular resistance concurrent with marked choleresis and the increasing bilirubin excretion into bile. Furthermore, laser-confocal fluorescence microangiography revealed that administration of XLHb evoked marked narrowing changes in sinusoids. The increasing excretion of bilirubin into bile occurred as early as 5 min after the start of XLHb, suggesting its rapid turnover in the liver. By contrast, liposome-encapsulated hemoglobin did not evoke these changes. These results suggest that liposomal encapsulation is required for development of hemoglobin-based blood substitutes that do not affect physiological functions of the liver.

HEMOGLOBIN-VESICLES PREPARED BY CONTROLLING THE INTERACTION OF HEMOGLOBIN AND PHOSPHOLIPID MEMBRANE

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In the preparation of Hb-vesicles(HbV) which encapsulate concentrated hemoglobin (Hb), intermolecular interaction of Hb with lipid bilayer membrane is important to control the number of bilayers (n) and the Hb concentration in Hb-vesicles ([Hb]in). In this paper, the zeta-potential of Hb molecules, the surface potential of the phospholipid membrane, the molecular mobility in bilayer membrane, the solution viscosity, and the solution pH are measured, and the factors which influence Hb encapsulation into the phospholipid vesicles are determined from n and [Hb]in.

Hemoglobin was easily isolated from outdated red blood cells (Hokkaido Red Cross Blood Center) using our original processes [1]. HbV was prepared in the same manner as previously reported in the literature [2]. The encapsulated solution of carbonylhemoglobin (ca. 38g/dl) contained pyridoxal 5'-phosphate (18mM) as an allosteric effector and homocysteine (5mM) as a metHb reductant. The lipid bilayer was composed of Presome PPG-I(Nippon Fine Chem.) and α-tocopherol.

The structure of a Hb-vesicle was roughly defined by the radius of the vesicle, the number of bilayers (n), the average molecular area of the lipid molecules (S), the thickness of the bilayer, the distance between bilayers, and the concentration of Hb in the inner aqueous phase of a Hb-vesicle ([Hb]in) . The weight concentration ratio of [Hb]/[lipid] indicates the quality of HbV; the higher ratio means the higher oxygen carrying capacity of one vesicle. They were measured or calculated according to the method in the literature[3].

The n of the resulting vesicles decreased when the surface potential of the layer became negatively high because of electrostatic repulsion between the bilayers. On the other hand, with changing the zeta-potential of Hb(pI;7.0) from positive to negative, the [Hb]in of the Hb-vesicles showed a precipitous fall because the electrostatic repulsion between Hb and the negatively charged bilayers prevented the access of Hb molecules to the vicinity of the bilayer membrane. As a result, the [Hb]/[lipid] shows a maximum at the preparation pH of 7.0. The low mobility of lipids in the bilayers provided the smaller number of bilayers, and [Hb]in decreased slightly with increasing viscosity of the Hb solution. The lower temperature results in the higher [Hb]/[lipid] .

From the point of metHb formation, it is well-known that the lower pH leads to the faster rate of metHb formation. The pH of the inner aqueous phase of HbV should be 7.4 after the preparation of HbV at 7.0. However, it is very difficult to adjust the inner pH by changing pH of the outer phase after the preparation of HbV because of the low membrane permeability of ions. We proposed the use of CO_2 which can permeate through the bilayer membrane. The preparation of HbV was carried out using the concentrated Hb solution, of which pH was reduced from 7.4 to 7.0 by CO_2 dissolution. After the preparation of HbV CO_2 degassingwas carried out to control the inner pH to 7.4 to enable both the high [Hb]/[Lipid] ratio and the lower rate of metHb formation.

Refs. [1] Protein Expression Purif., 4, 563-569 (1993). [2] Biotechnol. Prog., 12, 119-125 (1996). [3] Langmuir, 12, 1755-1759 (1996).

DIFFERENCES IN OXYGEN TRANSPORT AND PHYSICAL PROPERTIES BETWEEN CELLULAR AND ACELLULAR HEMOGLOBINS

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Hemoglobin (Hb)-based oxygen carriers have been developed vigorously as red cell substitutes. Modified hemoglobins (intermolecular crosslinked Hb, intramolecular crosslinked Hb, polymer-conjugated Hb) are acellular hemoglobins, and currently under the way in clinical tests. However, some problems of cell-free Hb have been pointed out such as vasoconstriction, autoregulation, low viscosity etc. On the other hand, we have developed encapsulated hemoglobin such as Hb-vesicles (HbV) which encapsulate a purified and concentrated Hb solution with phospholipid bilayer membrane. They are expected to overcome the problems of cell-free Hb.

The properties of those oxygen carriers are compared. All of them have the similar oxygen affinity comparable to RBC and controllable. The solution viscosity of HbV is high due to the aggregation, but the surface modification of the HbV with polyethylene glycol chains suppressed the aggregation, resulting in the solution viscosity similar to that of blood. On the other hand, the Hb solution shows the viscosity lower than RBC. The permeability of the membrane filter depends on the particle size. Intramolecular crosslinked Hb has the same size as that of Hb (ca. 5 nm). While the size of the HbV is determined from the final pore size of the membrane filter during the extrusion procedure. In this experiment, the size of the vesicles is controlled to 250 nm in diameter. Such a difference was clearly reflected on the filter permeability of the isopore membrane filters.

Kinetic parameters of oxygen binding concludes that the acellular Hb shows fast binding and releasing rates in comparison with encapsulated types such as HbV and RBC. The rates of the HbV are in between those of cell-free Hb and RBC. This should be due to the low diffusion of oxygen in the high concentrated Hb solution (36 g/dL) encapsulated in the cell, and HbV has the larger surface area than RBC. The detail analyses of the dynamics clarified the biphasic profile of oxygen binding in cellular Hbs.

Such differences should come from the structure and size of those oxygen carriers, and they should also cause the different physiological responses of them. Namely, HbV having the particle size of ca. 250 nm should not be able to permeate through the vascular wall, whereas Hb can permeate to reach smooth muscle and trap nitric oxide from endothelial cells, resulting in the inhibition of its vasorelaxation.

INVOLVEMENT OF NITRIC OXIDE (NO) IN THE OXIDATION OF OXYHEMOGLOBIN AND OXYMYOGLOBIN BY NITRITE

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We devised an improved NO-selective electrode system (including a NO-selective electrode with Pt/Ir alloy, and a control electrode with a carbon), which enabled us to measure a small amount of NO in phosphate solution, continuously and quantitatively. By using this electrode system, we found that NO was generated during the oxidation of oxyhemoglobin and oxymyoglobin by nitrite. [Method] Human hemoglobin was obtained from freshly obtained bloods, and was purified by the conventional method. Horse heart myoglobin was reduced to deoxymyoglobin by dithionite, and was also purified. Oxyhemoglobin (160 μM in heme) and oxymyoglobin (65 μM in heme) were reacted with various concentrations of nitrite. The production of NO was monitored continuously by an improved NO-selective electrode system (Inter Medical Co). The calibration curve for NO was obtained by measuring NO produced from NOC-5, i.e., 3-[2-hydroxy-1-(1-methyl-ethyl)-2-nitrosohydrazino]1-propanamine, with the above-stated NO-selective electrode system. [Results and Discussion] When oxyhemoglobin was reacted with 500 μM nitrite, NO was generated quickly at the initial lag phase of the oxidation of oxyhemoglobin by nitrite and decreased gradually during the second burst phase of the reaction, while the oxidaiton of oxyhemoglobin by nitrite proceeded in a sigmoidal manner including the initial lag phase and second burst phase. According to the increase of nitrite added (1 mM or 2 mM), the amounts of NO generated at the initial phase increased, being good accordance with the increased rate of the oxyhemoglobin oxidation. On the other hand, when 500 μM nitrite was added in the solution of oxymyoglobin (65 μM), oxymyoglobin was oxidized to metmyoglobin in a sigmoidal manner. During the reaction, NO was generated quickly at the initial lag phase, and reached its peak (NO approximated to 30 μM), before the burst oxidation of oxymyoglobin occurred. Then, NO was gradually decreased. These results suggest that NO is involved in the oxidation of oxyhemoglobin and oxymyoglobin by nitrite, though the oxidation of these hemoproteins was shown to proceed sigmoidally, and its reaction mechanism has not been clarified. Our present results demonstrated that the NO-selective electrode system can be available for the continuous and quantitative measurement of NO produced in vitro and in vivo.

SYNTHESIS AND PROPERTIES OF AN OLIGOMERIC RECOMBINANT HEMOGLOBIN PRODUCED USING HETEROBIFUNCTIONAL CHEMICAL CROSSLINKING

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The objective of our work was to prepare a covalent oligomer of recombinant hemoglobin so we could test the role of hemoglobin molecular size in hypertension and esophageal dysmotility. We used the amino- and sulfhydryl group reactive heterobifunctional chemical crosslinker N-(γ-maleimidobutyryloxy)-sulfosuccinimide ester (sulfo-GMBS), to produce a mixture of oligomeric hemoglobins in 70 % overall yield. In the first reaction step, surface lysine residues of rHb1.1 were reacted with sulfo-GMBS. This activated "core" protein was isolated and reacted with excess rHb1.1[diαK158C], a mutant recombinant hemoglobin containing a single surface sulfhydryl residue. The end product molecular size distribution was controlled by manipulating reaction times and the stoichiometries of reactants. The desired oligomeric hemoglobin fraction was isolated by ion exchange chromatography and tangential flow ultrafiltration. The selected fraction was a mixture, with the number of hemoglobins in the covalent assembly ranging from n = 3-7, distributed about the predominant n = 5 oligomer. We therefore refer to this material as pentahemoglobin (pHb). The equilibrium oxygen binding parameters for pentahemoglobin at 37 °C and pH 7.40 are almost identical to the corresponding parameters for the starting hemoglobins (p50 = 32 Torr, N_{max} = 2.1), indicating negligible changes in oxygen delivery properties. The effect of pHb was compared to the mono-hemoglobin rHb1.1 (350 $mg.kg^{-1}$ iv) in conscious rats. The increase in mean arterial pressure (MAP) observed following administration of pHb was substantially less than that seen with rHb1.1. This reduction in response did not appear to be due to faster clearance of pHb relative to rHb1.1. Pentahemoglobin had a circulating half-life of 4.3 hr at this dose compared to 2.7 hr for rHb1.1. The effect of pentahemoglobin (500 $mg.kg^{-1}$ iv) on lower esophageal sphincter (LES) tension was compared to control rHb1.1 in anesthetized opossums. Pentahemoglobin caused a decrease (ca. -10 Torr) in preswallow LES tension, whereas rHb1.1 caused a significant increase (27 Torr). Pentahemoglobin also caused a negligible increase in LES tension during swallows whereas LES tension during swallows rose substantially with rHb1.1. However, pHb caused significantly more abnormal swallows than rHb1.1. These results indicate that an increase in molecular size can alter the magnitude of physiological responses following hemoglobin administration.

INCREASED BLOOD VISCOSITY DURING EXTREME HEMODILUTION IMPROVES MICROVASCULAR TISSUE PERFUSION

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Maintenance of tissue perfusion is critical to tissue viability. The decrease of whole blood viscosity during moderate hemodilution causes an increase in cardiac output which translates to an increase in tissue perfusion and oxygen delivery to the microvasculature. However, at higher levels of hemodilution (> 60% reduction in systemic hematocrit) the number of perfused capillaries declines and tissue perfusion is compromised. Using the awake hamster skinfold model, we investigated whether increasing blood viscosity at high levels of hemodilution would restore tissue perfusion. The first group of animals (low viscosity) were subjected to a 75% isovolemic exchange transfusion with 6% dextran 70 solution. In the second group (high viscosity), 60% isovolemic exchange transfusion was performed with the same solution followed by an isovolemic exchange transfusion with a high viscosity solution of 6% dextran 500 solution to the 75% exchange level. Systemic parameters of heart rate, blood pressure and blood gases were evaluated along with microvascular measurements of arteriolar and venular diameter, blood flow rate, and functional capillary density. Colloid osmotic pressure and blood viscosity were also measured after the experiment.

All animals survived the high viscosity protocol; however only 60% of the low viscosity protocol animals were able to maintain a blood pressure above 40 mmHg for 1 hr after the completed exchange transfusion, these animals were excluded from the analysis. After the 75% exchange, blood pressure was maintained in the high viscosity group to 94% from baseline; however the low viscosity group experienced a drop to 63%. A 33% arteriolar dilation and no change to the venular diameter was observed in the high viscosity group. Low viscosity exchange caused a 15% venular constriction and no effect on arteriolar diameter. The high viscosity solution maintained functional capillary density to 89% of baseline, within its natural fluctuation of -13%, while the low viscosity solution could only perfuse 33% of the capillaries. Viscosity of blood after the 75% exchange protocols was 1.7 in the low viscosity group and 2.8 in the high viscosity group. The colloid osmotic pressures were no different between groups.

Results show that increased blood viscosity during extreme hemodilution is very effective in maintaining tissue perfusion. A likely mechanism for the microvascular response is that blood viscosity is necessary for shear stress dependent vasodilation through the release of endothelial relaxing factors such as nitric oxide and prostacyclins.

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PAST, PRESENT, AND FUTURE PROSPECTS OF BLOOD SUBSTITUTES

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Safe blood substitutes, which are stored on a shelf under ambient conditions and easily utilized at any time and place, are essential to medical care in the next generation. Oxygen carrying metal chelates, modified hemes, hemoglobins, and encapsulated hemoglobins have been studied for a long time in both Japan and North America with the aid of the superior technology, in addition to the extensive clinical trials of the perfluorocarbon emulsion that took place in Japan between 1975 - 1982. Since 1992 in the United States, clinical tests of red cell substitutes have already been started and a few are now in Phase III testing. It seems to be only a matter of time before red cell substitutes will become available. The Society of Blood Substitutes, Japan, first met in 1993 with an eye to increase the number of researchers and funds available for blood substitute studies. Under these circumstances, the "VII International Symposium on Blood Substitutes" is to be held in Tokyo in 1997.

Acellular hemoglobin(Hb) or modified Hb such as crosslinked Hb, polymerized Hb, polymer-conjugated Hb, and recombinant Hb, and the clinical results will be summarized and their characteristics and properties described. The intramolecularly crosslinked Hb is prepared by crosslinking between α,α-units with diaspirin. This procedure satisfies the large-scale production and sterilization by heat treatment. Furthermore, crosslinking prevents dissociation into $\alpha\beta$ dimers, extends the half-life during circulation, and enables an appropriate P_{50} value. This is now in Phase III clinical trials, the vasoconstriction issue remaining. Recombinant Hb is also genetically designed from the same strategy and is now in Phase II. On the other hand, polymerized Hb could be intermolecularly crosslinked with glutaraldehyde to increase the size and to reduce the oncotic pressure, and is now in Phase III. Polyoxyethylene chains are conjugated to human or bovine Hb to prepare polymer-conjugated Hb with high molecular weight. They have completed Phase I.

In Japan, cellular Hb has been developed on the basis of molecular assembling science and technologies. Hb-vesicles, which encapsulate concentrated Hb with a phospholipid membrane, will be one of the prime candidates for use in the next generation of Hb-based oxygen carriers. The size of the vesicle (ca. 250 nm) is extremely large in comparison with that of the Hbs (5nm). The solution viscosity, oncotic pressure, P_{50}, etc. are controllable like those of blood. Discussing the differences between cellular and acelluar structures relating to the physical or physiological properties is very useful for designing the red cell substitutes with high efficacy and high safety as well as reconsidering the importance of the red blood cell's structure.

Totally synthetic oxygen carriers have been developed in our group. Lipidheme-vesicles or lipidheme-subnanospheres are vesicles or subnanospheres, respectively, of which the surface synthetic heme derivatives, lipidhemes, are incorporated as reversible oxygen-binding sites. Recently, we have also found eight heme derivatives conjugated to recombinant albumin having an oxygen-carrying capacity larger than Hb. The other new products including platelet substitutes, recombinant products, PFC, etc. and new methods including evaluation techniques, manufacturing process, etc. are introduced for the development of blood substitutes in the coming century.

The assessment of oxygen transport capacity of NRC in 70% blood exchange rats

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We investigate that the Neo Red Cell (NRC) effectively transports oxygen in vivo as blood substitute. We carried out 70% exchange transfusion for rats with NRC of various Hb concentrations, and investigated the optimum concentration of NRC.

PURPORE: 1) Oxygen dissociation curve of RBC show "S" for including 2,3-DPG as an allosteric effector, and oxygen saturation is over 95% when oxygen partial pressure is 100torr. The other side, NRC showed a hyperbolic curve for including inoshitol hexaphosphate(IHP) as an allosteric effector. Therefore, oxygen saturation is over 95% when oxygen partial pressure is over 300torr. We examined whether the oxygen supply with NRC for rats changed by using an oxygen mask.

2) We examined whether the oxygen transport capacity of NRC changed by including IHP as an allosteric effector.

3) At the basis of the result of 1) and 2), we evaluated Hb concentration (4,5,6,or 7%) of NRC required to have oxygen transport capacity similar to RBC.

METHOD: NRC was used with diluting 5% bovine albumin in saline. As control group, 5% bovine albumin in saline and rat's RBC with diluting 5% bovine albumin in saline (Hb concentration 13%) was used. Rats(Crj:Wistar,♂,290-420g) were anesthetized with pentobarbital sodium(1.25ml/kg). We cannulated into carotid artery (for drawing line), femoral artery (for blood pressure line), and femoral vein (for transfusing line). We carried out blood exchange with each sample until exchange rate was 70%.

RESULT: 1) The lactate value after exchange transfusion was three times higher than pre-exchange transfusion, when we did not use oxygen mask for rat. But, we suppressed increase of lactate when we used oxygen mask and kept 300torr oxygen partial pressure.

2) Despite of existence of IHP, NRC Kept enough oxygen transport capacity. But, V-O_2 of IHP free NRC was 25-30torr(oxygen saturation of NRC is 80%), so that NRC could not keep oxygen transport capacity under severer condition.

3) NRC (Hb conc. 4%) group were shown respiratory acidosis and a drop of blood pressure and heart rate, and did not keep enough oxygen supply. But, RBC and NRC (Hb conc. over 5%) group were shown stability after exchange transfusion.

CONCLUSION: We concluded that the Hb concentration of NRC needs over 5% for functioning as effective blood substitute in vivo, and using oxygen mask was effective when we did 70% blood exchange with NRC for rats.

EFFECT OF NEO RED CELLS ON HEMORRHAGIC SHOCK

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Liposome encapsulated hemoglobin, Neo Red Cells (NRC), shows low viscosity (2cP) and high oxygen transport capacity (P_{50}: 45-55 Torr). In this study, we examined the effects of NRC on hemorrhagic shock. Nineteen mongrel adult dogs weighing 8.0 to 11.0 kg were used. In 6 dogs (Group 1), NRC was substituted for blood, while avoiding shock by combining exsanguination with NRC transfusion. To prepare a mild shock model, NRC was administered immediately after inducing shock by exsanguination through the vein, then this was repeated three times in 6 dogs (Group 2). To prepare severe shock model, NRC was administered in 7 dogs (Group 3) left untreated for 30 minutes after inducing shock, then this was repeated three times. These groups were compared. In Group 3, shock was induced at a smaller volume of exsanguinated blood than in Groups 1 and 2. In Groups 1 and 2, administration of NRC at a dose equivalent to the volume of exsanguinated blood improved shock, and circulatory kinetics showed normovolemia. However, in Group 3, a dose of NRC 1.6-fold the volume of exsanguinated blood was required, but hypovolemia persisted. In Groups 1 and 2, peripheral vascular resistance (PVR) decreased after NRC administration. However, PVR was increased in Group 3. Cardiac index (CI) was increased in Groups 1 and 2, while it was decreased in Group 3, resulting in progression of heart failure. In other words, administration of NRC with a low viscosity improved circulatory kinetics in Groups 1 and 2, but not in Group 3. Concerning oxygen kinetics, there were no increases in oxygen requirement in Groups 1 and 2. The low viscosity of NRC reduced vascular resistance, increasing CI. Therefore, there were no increases in arteriovenous difference of oxygen content per hemoglobin (AV/Hb) for NRC. In Group 3, oxygen requirement and oxygen consumption were increased, while CI was decreased. However, NRC compensated for the decrease in CI with an increase in AV/Hb by decreasing SvO_2 and increasing the oxygen transport efficiency to cope with increased oxygen requirement. However, erythrocytes could not increase AV/Hb, and could not cope with increased oxygen requirement. Therefore, NRC was very effective in treating henorrhagic shock with severe peripheral circulatory failure.

NATIONAL BLOOD PROGRAM IN THE PEOPLE'S REPUBLIC OF CHINA

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China is a developping country with the largest population in the world. There are 4 municipalities, 23 provinces and 5 autonomous regions under the Central Gavernment. The governments are derectly in charge of the blood programs in China, cooperating with Chinese Red Cross Society and Chinese Society of Blood Transfusion. Central Government, through the Ministry of Public Health, is responsible in national basis and local governments for their own areas.

Today, there are about 170 blood centers in China. More than 10 million units (200ml per unit) of whole blood were collected annually by the centers, of which about 50% were from paid donors, 40% from voluntary donors and 10% from voluntary nonremunerated donors. About 30% blood were processed into various blood components for clinical use.

Besides the blood centers, there are over 200 plasma collecting centers in China. About 2000 tons of plasma were collected annually for production of plasma derivatives in 18 manufactories. The main plasma derivatives produced were albumin, intravenous immunoglobulin, factor Ⅷ and prothrombin complex concentrate.

Recent years, the Ministry of Public Health made great efforts in the safest possible supply of blood and plasma derivatives. Several new laws and decrees have been promulgated, which included the Administrative Decree for Blood Services, Basic Standards for Blood Centers, Administrative Decree for Manufacture of Plasma Derivatives, Standards for Plasma Collecting Centers and Standards for Hospital Transfusion Departments. National and local Blood Quality Insurance Committees have also been founded to put these laws and decrees into effect. The Blood Donation Law is being drawn up. As a result, the voluntary nonremunerated donation is increasing and the safety of the blood and plasma derivatives used in clinical also increasing.

Blood Program and the possibility of applying red cell substitutes in Indonesia

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Indonesia, a country of 13.667 islands of which separated by sea has made tremendous improvement during the past decades, including health sector, proved by the statistical data that in 1965, 60% of people were living below the poverty line, while in 1993, only 13,37%. Infant Mortality Rate has decreased from 145/1000 live births to 58/1000, while life expectancy has increased from 45,7 years to 61,5 years.

The existing Health Centers were 1.227, have been increased into 6.227, with 18.946 Sub Health Center and Integrated Health Services.

However, this fast movement could not be followed by the establishment of Blood Services, especially in the very remote area due to geographical problems, from 337 Public Hospital, there are still 129 type D hospital without surgery room and blood bank facilities, while Red Cross could not establish the Blood Transfusion Service (BTS) in those area due to the very low demand of blood and financial limitation, although there are 153 BTS widely spread within the country with approx 900.000 donations/year. This condition reflected in the remain persistent Maternal Mortality Rate, from 450/100.000 in 1986 to 420/100.000 in 1996. The traffic accident have also increased, although this reflected the increasing standard of living.

Lack of blood due to geographical problem, will be diminished by providing Red Cell substitutes in those area to prevent bleeding <1.200 ml as the sole emergency remedies, while for larger amount of haemorrhage the used of artificial oxygen carriers can be combined with other indicated blood products.

THE ROLE OF BLOOD SUBSTITUTES IN EMERGING HEALTHCARE SYSTEMS

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Blood transfusions play a major role in the healthcare systems of the developed world. Beyond the obvious applications in trauma, surgical hemorrhage and other emergencies, the availability of safe, compatible blood enables surgical procedures such as organ transplantation and makes cardiopulmonary bypass a routine component of coronary artery bypass and other cardiac surgeries. Technical advances, information dissemination and excellent medical training available now in all but the most remote areas of the world mean that such sophisticated medical practices are or soon will be available to the majority of the world's population. For many reasons, it is unlikely that the supply of blood for transfusion will keep pace with this progress. High prevalence of endemic diseases and poor general health disqualify many donors, and blood banking infrastructures are not developed well enough in most emerging countries to make optimal use of the blood that is collected. Based on the per capita blood use in the U.S. of 1 unit per 20 persons per year, and the estimate of 90,000,000 units of blood collected per year worldwide, the global annual deficit is at least 135,000,000 units per year. While staggering, this number is a minimum since the known and perceived dangers of blood transfusion presently restrict its use, and aging of the population will contribute to an increasing demand for blood services. In this context, "blood substitutes" can contribute significantly to medical practice worldwide if they meet the essential criteria of safety, efficacy and low cost. In particular, to be used in practice, the cost burden to the healthcare system of these new products must be affordable within the system. The availability of such products will greatly influence how blood services evolve in developing countries.

IMMUNOLOGICAL INVESTIGATION IN RATS HIGH GRADE BLOOD REPLACEMENT BY NEO RED CELLS

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[INTRODUCTION] A liposome encapsulated hemoglobin named Neo Red Cell (NRC; TERUMO) has been developing as artificial blood substitute for hemorrhagic shock. We made a total body rinse model in rats by a high grade of blood exchange with NRC and investigated the xenogenic immunological reaction.

[MATERIALS AND METHODS] Brown Norway rats were used. We extracted blood from rats at a settled rate, and transfused NRC at same rate. These manipulations were repeated until the exchange rate reached about 80-85%. After blood exchange, guinea pig heart were transplanted in the necks of BN rats and the manner of acute rejection was observed. Complete blood count, hemogram, C3, CH50 were measured during the experiments.

[RESULTS] Hematocrit (Hct) decreased from 43.5±1.9% to 7.1±1.5%, and the rate of exchange was calculated at 83.5±3.8%. Hct had not recovered 24 hrs after blood exchange (8.5±1.9%). Meanwhile white blood cells decreased from 13230±2181 to 6759±2046 / μL and fell to 5350±1915/ μL 3~4 hrs later, and 3975±877/ μL after 10~16 hrs. Later exchange, lymphocytes decreased from 10895±2243 to 5667±1483 / μL and decreased still more to 2242±355 / μL 16 hrs later, but neutrophil decreased from 2648±1634 to 1168±1116 / μL but recovered 1547±635 / μL 16 hrs later. Relatively immediately after blood exchange C3 and CH50 values decreased markedly and remained unchanged 10~16 hrs later. Graft survival of GP—›BN Hx with untreated control group was 11.4±2.1 min by hyperacute rejection (HAR). However, graft survival after blood exchange was prolonged with a mean of 875±183 min. Histological findings of grafted heart showed severe injury of intima and intramuscular hemorrhage in the control, but there were no signs of HAR except infiltration of neutrophil in the cases sacrificed 4 hrs after blood exchange. It was observed that complements were partially activated in the cases which were sacrificed after graft beating ceased.

[DISCUSSION] In this high grade model of blood exchange, complement activity is markedly suppressed even 16 hrs after exchange. It is well known that existence of preformed antibodies and activation of alternative pathway of complement plays an important role in the onset of HAR. Although HAR occurred in this model, graft survivals were significantly prolonged by high grade blood replacement by NRC. It was suggested that neutrophil also played a crucial role in delayed rejection in this model.

SELF-ASSEMBLED LIPIDHEME VESICLE: TOTALLY SYNTHETIC ARTIFICIAL RED CELL

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Amphiphilic porphyrinato-irons (lipidhemes) have been synthesized and utilized as totally synthetic artificial red cells, *i.e.* phospholipid vesicles embedded lipidhemes,[1] lipidheme-microspheres[2] and human serum albumin incorporated hemes.[3] All systems can bind and release oxygen reversibly under physiological conditions (pH 7.4, 37°C) like as hemoglobin and their O_2 transporting abilities *in vitro* and *in vivo* have been studied.

We have recently found that lipidheme itself forms some assembling structures in aqueous media (fibers, ribbons, sheets, *etc.*). For example, lipidheme having four dialkylphosphocholine groups produced spherical unilamellar vesicles, which can bind oxygen. This is the first example of totally synthetic red cell substitute composed of only amphiphilic heme aggregates.

The lipidheme was easily dispersed in water by a sonication procedure to give a red-colored solution, which did not change for several months at the ambient conditions. From electron microscopy (TEM), spherical unilamellar vesicles with diameter of 100 nm were clearly observed. The thickness of the membrane was estimated by cryo-TEM to be 92 nm, corresponding double the length of the molecule.

The lipidheme vesicles with 3-fold excess molar of dodecylimidazole as an axial base, were prepared by same procedure. The ferric hemin of the vesicles was reduced to a ferrous state by adding small excess amount of ascorbic acid under nitrogen atmosphere. The visible absorption spectra of the deoxy form (λ_{max}: 434, 536, 558 nm) changed immediately to oxygenated species (λ_{max}: 432, 540 nm) after exposure to oxygen. This spectral changes were reversibly dependent on the O_2 pressure. The O_2 adduct form shifted to the strong CO adduct after bubbling CO gas. The O_2 binding affinity ($P_{50}(O_2)$) was estimated to be 47 Torr under physiological conditions. The structure and O_2-binding behaviors of this self-assembled lipidheme vesicle will be discussed.

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NITRIC OXIDE AND BLOOD

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The circulating blood contains certain steady-state concentrations of nitric oxide (NO) in the plasma (~0.1 to ~1 μM), in order to maintain normal vascular tone and other appropriate conditions for the systemic and pulmonary circulation. This homeostasis of NO in the blood is maintained by delicate balance between its production by various forms of nitric oxide synthases (Ca^{++}/calmodulin-activated *c*NOS, cytokine-induced *i*NOS, and neurally-activated *n*NOS) and its scavenging primarily by hemoglobin (Hb) in the erythrocytes. Down- and upward deviations of the plasma concentration of NO from norm could wreak havoc in the circulatory system. Nitric oxide-generating reagents like nitroglycerin, nitroprusside, and nitrite as well as cytokines, which might be derived from microbial infection, NOS inhibitors, and blood transfusion sometimes contribute to such situations. Constriction of blood vessels, resulting in elevated blood pressure, vascular adhesion of leukocytes, aggregation of platelets have been observed when the plasma concentration of NO was reduced. Plasma NO constantly diffuses into the erythrocytes, and immediately reacts with Hb, which acts as a bottomless NO scavenger, and is eventually oxidized to bio-inactive nitrate.

As *oxy*Hb reacts rapidly with stoichiometric quantities of NO in vitro, it has been assumed that plasma NO is scavenged by rapidly reacting with *oxy*Hb to produce such oxidation products as *met*Hb and nitrate under physiological conditions. Then the so-formed *met*Hb is immediately recycled back to bioactive *deoxy*Hb by *met*Hb reductase in the erythrocytes. However, this widely accepted mechanism of NO scavenging by Hb does not appear to take place as the principal pathway for the NO scavenging in the blood, where the plasma concentration of NO (<1 μM) is substantially lower than the intra-erythrocyte concentration of *oxy*Hb (15 to 20mM heme). Instead, the plasma NO binds to *deoxy*Hb in the erythrocytes to form partially *nitrosylated* Hb, since the high affinity of NO for *deoxy*Hb (K_D = ~5*n*M) and their high kinetic association constant ($k_{on} = 2.5 \times 10^7\ M^{-1}s^{-1}$) far exceed the corresponding values expected for the reaction of NO with *oxy*Hb. The so-formed partially *nitrosylated* Hb is predominantly α-*nitrosyl*Hb, $\alpha(Fe\text{-}NO)_2\beta(Fe)_2$, which is found to be an efficient, low-affinity O_2 carrier. Although it is surprisingly stable, α-*nitrosyl*Hb is eventually converted to oxidized products, *met*Hb and nitrate, under aerobic conditions of the blood. *Met*Hemoglobin is, of course, recycled to bio-active *deoxy/oxy*Hb by the reductase.

We found that α-*nitrosyl*Hb, $\alpha(Fe\text{-}NO)_2\beta(Fe)_2$, can deliver O_2 to the peripheral tissues more efficiently than normal Hb, especially under acidic (<pH 7.4) conditions. This observation may point to the possibilities that NO not only acts a vasodilator, as well established, but also stimulates Hb to deliver O_2 to tissues more effectively, especially under local, pre-capillary conditions. This observation also explains the mystery that clinical treatments of inhaled NO for the newborn with pulmonary hypertension are accompanied with no *acute* adverse effect, though NO has a more-than-thousand-fold higher affinity for *deoxy*Hb than CO, the culprit of the CO poisoning. This observation also points to the practical possibility of rejuvenating the expired blood by treatments with NO, as it is erythrocyte membrane-permeable. Exogenous addition of 2,3-Diphosphoglycerate, the natural allosteric effector of the blood, which is depleted during storage, is not effective, as it is membrane-impermeable.

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EVALUATION OF THE OXYGEN TRANSPORTING CAPABILITY OF ALBUMIN-HEME IN A RAT SHOCK MODEL

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The oxygen transporting capability of Albumin-Heme (HSA-FeP), a totally synthetic oxygen carrier, was carried out in a rat shock model. HSA-FeP was reconstituted in 5% albumin (HSA-FeP group) and compared with 5% albumin alone (albumin group). The amount of FeP and HSA was 3.0mM and 5.0g/dL. The ratio FeP to HSA was 1 to 4. The viscosity of the vesicular suspension became 1.0 centipoise. P_{50} was regulated 30 Torr. Oncotic pressure was calculated to be 25 Torr. Systemic mean arterial pressure (MAP), arterial and venous blood gas (PaO_2 and PvO_2 respectively), abdominal aortic blood flow and tissue oxygen tensions of the renal cortex ($PtrO_2$) and skeletal muscle ($PtmO_2$) were monitored as indices of oxygen transport. To minimize the effects of native rat red blood cells, 80% of the estimated circulatory blood volume was exchanged with 5% albumin before the induction of shock. Then shock was induced by withdrawing 40% of the estimated circulatory blood volume followed by isovolemic infusion of HSA-FeP or albumin.

In the albumin group MAP was 30.9% of baseline after infusion whereas in the HSA-FeP group it recovered to 64.2% of baseline.
In the albumin group abdominal aortic blood flow was 66.8% of baseline after infusion whereas in the HSA-FeP group it recovered to 107.6% of baseline.
In the albumin group $PtrO_2$ and $PtmO_2$ was 37.9% and 28.0% of baseline respectively after infusion whereas in the HSA-FeP group they recovered to 58.3% and 43.8% of baseline respectively.
In the albumin group PvO_2 was 62.8% of baseline after infusion whereas in the HSA-FeP group it was to 40.5% of baseline.
At 30 minutes after infusion, none in the albumin group was alive whereas in the HSA-FeP group survival was 100%.

From these results we can conclude that cardiac function and peripheral perfusion was conserved in the HSA-FeP group due to its oxygen transporting capability. Concerning PvO_2, the higher value in the albumin group probably resulted from shunting of tissues due to the collapse of peripheral circulation.

THE OXYGEN TRANSPORTING CAPABILITY OF HEMOGLOBIN VESICLE EVALUATED IN A RABBIT SHOCK MODEL:EFFECTS ON THE SMALL INTESTINE

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The oxygen transporting capability of Hemoglobin Vesicle (HbV) was evaluated in a rabbit shock model. The hemoglobin concentration of Hemoglobin Vesicle was regulated to 10g/dL when dispersed in 5% albumin. The diameter was controlled to 257±81nm. Its P_{50} was regulated 32 Torr. Oxygen transport to the small intestine was monitored during shock and resuscitation. As indices of oxygen transport to the small intestine, blood flow of the superior mesenteric artery, gas analyses of venous blood from the superior mesenteric vein, intestinal mucosal pH and intestinal tissue oxygen tension were monitored. The fluids used for resuscitations were HbV dispersed in 5% albumin (HbValb) (Hb 10g/dl), 5% albumin (alb) and washed rabbit red cells dispersed in 5% albumin(RBCalb) (Hb 10g/dl). The results were compared. Shock was induced by withdrawal of 40% of the estimated circulatory blood volume followed by isovolemic infusion of each of the above mentioned fluids. The procedure was repeated twice.

In the alb group blood flow of the superior mesenteric artery was 93±22% of baseline after the second infusion whereas in the HbValb and RBCalb groups they were 103±49% and 93±23% of baseline respectively.
In the alb group systemic base excess (BE) was -18.9±1.5% of baseline after the second infusion whereas in the HbValb and RBCalb groups they were -4.7±1.9% and -0.4±1.8% of baseline respectively.
In the alb group venous oxygen tension of blood from the superior mesenteric vein (PvO_2) was 99±19% of baseline after the second infusion whereas in the HbValb and RBCalb groups they were 102±19% and 129±36% of baseline respectively.
Blood flow of the superior mesenteric artery was also similar between groups. However, systemic BE, intestinal mucosal pH and intestinal tissue oxygen tension were sustained significantly higher in the HbValb and RBCalb groups compared to the alb group. The small intestine is one of the most vulnerable organs in shock and from these results we can assume that intestinal perfusion was more optimal in the HbValb and RBCalb groups due to their similar oxygen transporting capabilities.

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