acid and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG at a molar ratio of 5/5/1/0.033 (Nippon Fine Chemical Co. Ltd., Osaka, Japan)]. The HbV was suspended in saline at the Hb concentration of 10 g/dl, and sterilized using filters (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], < 3%; [HbCO], < 2%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 28 Torr.

HbV Infusion and Procedure for Serum Clinical Laboratory Tests

All animal studies were approved by the Animal Subject Committee of School of Medicine, Keio University, and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication #85–23 Rev. 1985). The experiments were carried out using five male Wistar rats (264 ± 8 g; Saitama Experimental Animals Supply Co. Ltd., Kawagoe, Japan). They were anesthetized using diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg. All rats were housed individually in cages and provided with food and water *ad libitum* in a temperature-controlled room on a 12 h dark/light cycle.

Each rat's body weight was measured after 1, 3, 6, 9, and 12 months. At 12 months, the rats were anesthetized with 1.5% sevoflurane inhalation (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) using a vaporizer (TK-4 Biomachinery; Kimura Medical Instrument Co. Ltd., Tokyo). The animals were laparotomized and about 6 ml of blood was withdrawn from the caudal vena into a heparinized syringe for plasma biochemical tests. Then they were sacrificed by acute bleeding from the abdominal aorta. The organs were resected and were fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo) for staining with hematoxylin/eosin and Berlin blue. The withdrawn blood was centrifuged to obtain plasma, and the serum specimens were stored at − 80°C until routine clinical laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyltransferase (gamma-GTP), creatine phosphokinase (CPK), amylase, lipase, uric acid (UA), urea nitrogen (BUN), creatinine (CRE), total cholesterol, triglyceride (TG), free fatty acid (FFA), phospholipids, total bilirubin, and Fe^{3+}. The assay methods are described in our previous report [13].

For the comparative histopathological study, five male Wistar rats (31 weeks, body weight, 472 ± 8 g) were used.

Data Analysis

All control results and one-week observations after HbV infusion are taken from our previous report [8]. Differences between the control and a treatment group were analyzed using a one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. Changes were considered statistically significant if $p < 0.01$.

RESULTS

All five rats survived one year; they were apparently healthy. The body weight before infusion was 264 ± 8 g, which increased monotonously to 821 ± 75 g (Fig. 1). However, all rats became obese because of their confinement in small cages individually for one year.

Plasma biochemical analysis clarified that no abnormal values were detected that reflect liver functions such as AST, ALT, and LDH, and those of kidney such as UA and CRE (Fig. 2). In a previous report, lipase activity increased transiently at 1 and 2 days, but it was quite normal at one year.

Plasma lipid components, cholesterol, phospholipids, and triglyceride were significantly higher than the control values and those at 1–3 days after the bolus infusion of HbV in the previous report [8], as shown in Fig. 3. According to the data of the breeders, hyperlipidemia was confirmed for one-year-old rats housed in pairs in cages, not individually. Total bilirubin and iron concentrations were not higher at one year.

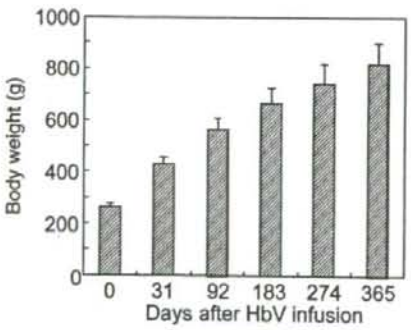

Figure 1. Changes in body weight of the Wistar rats during one year following intravenous bolus infusion of HbV at a dose rate of 20 ml/kg. All the rats were housed individually in cages and provided with food and water *ad libitum*.

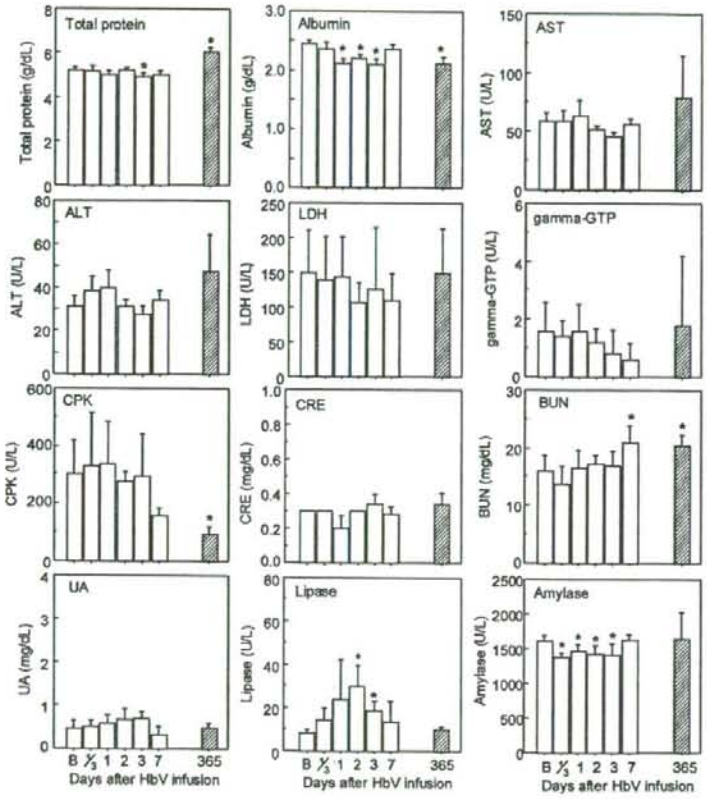

Figure 2. Changes in plasma biochemical parameters after intravenous bolus infusion of HbV into Wistar rats at a dose rate of 20 ml/kg. Data for 7 days (white bars) are cited from our previous report [8]. B, baseline.

No apparent anatomical abnormalities were detected in the lung (Fig. 4), liver, pancreas, heart, testis, stomach, intestine, kidney, muscle, bone marrow, or skin. One exception was that one rat showed an adenoma in the pituitary gland. Berlin-blue staining revealed considerable amounts of hemosiderin deposition in the spleen, pancreas, kidney, and adrenal glands. Rats that received no HbV also showed considerable amounts of hemosiderin in the same organs.

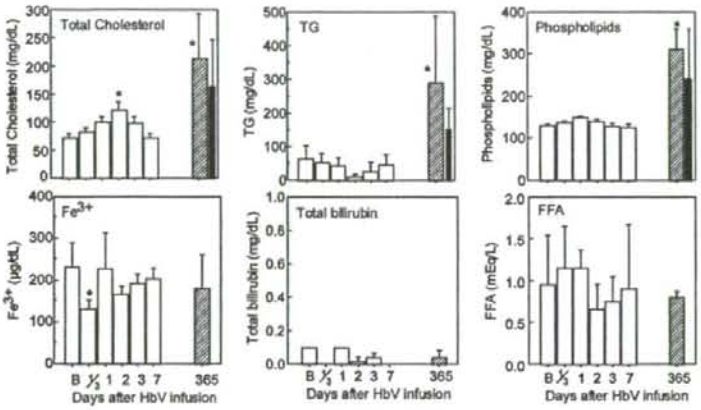

Figure 3. Changes in plasma levels of lipid components, iron, and bilirubin after intravenous bolus infusion of HbV into Wistar rats at a dose rate of 20 ml/kg. Data for 7 days (white bars) are cited from our previous report [8]. B, baseline. The values of black bars are from one-year-old rats housed in pairs in cages, not individually (cited from the breeder's data) [17].

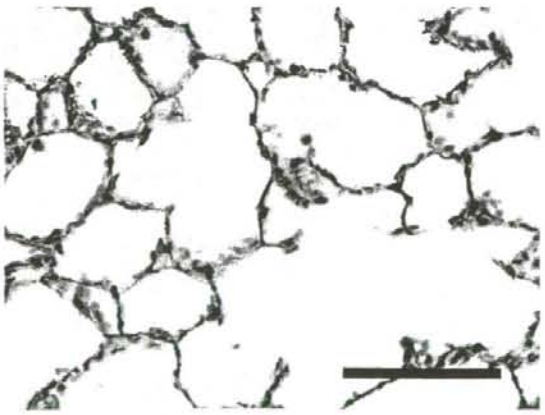

Figure 4. Histology of lung one year after bolus infusion of HbV stained with hematoxylin/eosin. Scale bar, 100 μm.

DISCUSSION

Our group has studied safety issues of HbV extensively [1,3,7–9,14,15]; no deteriorative or irreversible side effects have been observed after HbV infusion. Therefore, it was not surprising for us that all the rats survived one year after receiving intravenous bolus infusion of HbV (20 ml/kg) with no pathological abnormality in the lung, even though this was the first study to confirm one-year survival after HbV infusion. We conducted this experiment because it was communicated by Dr. A. Nosé that one lipid-emulsified perfluorocarbon (PFC) solution induced a chemical pneumonitis, causing death within one year (personal communication) [10]. In his recent review paper, he explains the mechanism that the unstable oxygen-carrying particles, such as PFC emulsion, would be covered with plasma proteins, and they would be trapped in the pulmonary capillary, resulting in the chemical pneumonitis [16]. On the other hand, in our experiments of HbV we did not observe any pathological abnormalities in the lung in one year. This should be due to the dispersion stability and blood compatibility of HbV achieved by the surface modification of HbV with PEG chains.

Serum biochemical analyses showed overall normal values. Albumin, AST, ALT, LDH, gamma-GTP, CRE, BUN, UA, lipase, and amylase were similar to the baseline values. The significant decreases in CPK and the significant increases in total proteins should be attributed to aging according to background data of the animal breeders [17,18]. In the previous report, plasma levels of lipid components increased transiently: they were liberated from macrophages that entrap HbV. They subsequently showed the maximum value at 1 or 2 days, and reverted to the control level at 7 days [8]. On the other hand, the present study showed significantly higher lipid levels in the rats at one year. All the rats gained weight significantly and apparently became obese from their confinement in small cages individually for one year with *ad libitum* access to food and water. The body weights of rats (821 ± 75 g) that received HbV were significantly higher than those of normal 52–week-old Wistar rats of the breeders (703 ± 61 g, caged in pairs [17]; 490 ± 50 g [18]). It seemed that the plasma lipid increase at 1 and 2 days after the HbV infusion had no impact compared with the levels of hyperlipemia because of obesity of the old rats.

Anatomical examination clarified that there were no apparent abnormalities in the vital organs including the lung. However, one rat showed an adenoma in the pituitary gland. This can result from aging, according to the literature [19–21] and information obtained from the animal breeders [17], describing that aged rats often show pituitary adenoma. Berlin-blue staining showed considerable amounts of hemosiderin

deposition in the spleen, liver, pancreas, kidney, and adrenal glands. It has been reported that hemosiderin deposition in the rat increases with age, especially in the liver and spleen [22–25], and pancreas in corpulent rats [26]. Actually, rats that did not receive HbV (31 weeks old) also showed considerable amounts of hemosiderin in those organs. Therefore, we speculate that hemosiderin deposition in this case is attributable to aging.

In conclusion, all rats survived for one year after bolus infusion of HbV at the dose rate of 20 ml/kg with no deteriorative side effects. All significant changes that were observed are attributable to the age and obesity of the rats. Further study is necessary to observe survival after a clinically relevant dose of HbV infusion such as that administered for resuscitation from hemorrhagic shock or a high level of blood exchange.

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Hemoglobin-Vesicles as a Transfusion Alternative

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Abstract: Phospholipid vesicles or liposomes encapsulating purified and concentrated human hemoglobin (Hb-vesicle, HbV) have been developed as a transfusion alternative. They are void of blood-type antigens and infectious viruses; they are stable and suitable for long-term storage. The cellular structure of HbV (particle diameter, ca. 250 nm) prevents direct contact of Hb with the blood components and the endothelial lining shielding cells from the side effects of Hb molecules. Microcirculatory observations show that the cellular structure of HbV is important to control reactions with endothelium-derived vasorelaxation factors. Animal studies of extreme hemodilution and resuscitation from hemorrhagic shock attest to the sufficient oxygen transporting capacity of HbV. Studies of biodistribution and metabolism reveal that HbVs are captured eventually in the reticuloendothelial system, and degraded within one week. In a joint collaboration partnership of academia, a biotech venture company and a corporation, we plan to produce HbV with good manufacturing practices, and to start preclinical and, finally, clinical trials within a few years.

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Keywords: Artificial oxygen carriers; Hemoglobin-vesicles; Transfusion alternative

1. INTRODUCTION

Hemoglobin (Hb)-based O_2 carriers (HBOCs) have been developed for use as a transfusion alternative. Some of them are undergoing clinical trials [1,2]. Advantages of the HBOCs include the absence of blood-type antigenicity and infectious pathogens, and better stability for long-term storage than RBC transfusion [1,3]. Phospholipid vesicles or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) have been developed as O_2 carriers [1–6]. The HbV cellular structure (particle diameter, ca. 250 nm) has characteristics that are similar to those of natural RBCs because both have lipid bilayer membranes that prevent direct contact of Hb with blood components and the endothelial lining. Reasons for the Hb encapsulation in red blood cells (RBCs) include: (1) a decreased high viscosity of Hb and a high colloidal osmotic pressure; (2) prevention of Hb removal from the blood circulation; and (3) preservation of the chemical environment in RBCs such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs), many side effects of molecular Hb have become apparent. These side effects of molecular Hb imply the importance of the cellular structure.

After the unforgettable tragedies of HIV transmission because of the distribution of non-pasteurized plasma-derived products and the Great Hanshin Earthquake in 1995, the Japanese government aggressively pursued development of artificial blood. In 1997, the Ministry of Health and Welfare declared a framework of scientific research grants intended for developing a substitute for three major blood components: artificial red cells, artificial platelets, and artificial antibodies. The most promising artificial red cell in Japan should be HbV. The in vitro and in vivo studies of HbV in collaboration with Waseda-Keio and other research institutes have revealed sufficient O_2 transporting efficiency comparable to RBCs, safety in terms of blood compatibility [7,8], and prompt degradation in the reticuloendothelial system, all of which makes us confident about advancing to further development of HbV. In this review paper, we demonstrate some important results that show the safety and efficacy of HbV as a transfusion alternative.

2. EFFICACY OF HBV SUSPENDED IN AN ALBUMIN SOLUTION

One HbV contains about 30,000 Hb molecules. For that reason, the suspension of HbV does not show colloid osmotic pressure (COP). Accordingly, the

infusion of a large amount of HbV suspension requires the addition of a plasma expander such as human serum albumin (HSA). Surface modification of HbV with polyethylene glycol (PEG) is effective to prevent intervesicular aggregation in the presence of HSA by the steric hindrance of PEG chains (Fig. 1). This measure guarantees homogeneous dispersion of HbV in the microcirculation. When HbV is suspended in 5-g/dl HSA, the COP and the viscosity of the suspension are respectively regulated to 20 mmHg, and 3 cP, which are comparable with those of blood.

A clear result of O_2 transporting ability of HbV was obtained using the extreme hemodilution with HbV suspended in HSA [9–11]. The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex and skeletal muscle; then the blood flow rate in the abdominal aorta was measured using the pulsed Doppler method. Hemodilution with albumin alone engendered marked reductions in mean arterial pressure and renal cortical O_2 tension. Finally, all the rats died of anemia. On the other hand, hemodilution with HbV suspended in HSA sustained both blood pressure and renal cortical O_2 tension: all the rats survived. These results demonstrate that HbV has sufficient O_2 transporting capability. It has been confirmed in a rat model that HbV suspended in recombinant HSA (rHSA) is effective as a priming solution for cardiopulmonary bypass circuit (about 50% hemodilution). Quite recently, we

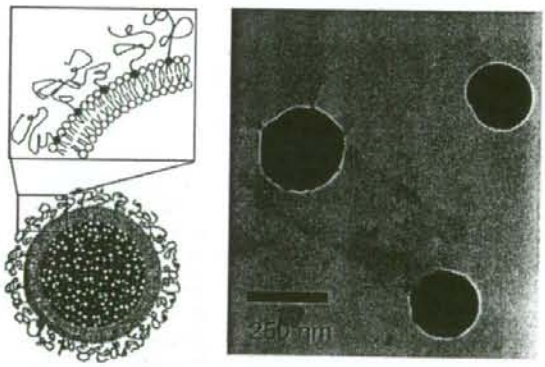

Figure 1. Highly purified and concentrated human-derived Hb solution is encapsulated in phospholipid vesicles (liposomes). One particle contains about 30,000 Hb molecules. The outer surface of one HbV is modified with about 1,500 polymer chains of PEG that ensure the dispersion stability of HbV during storage and during circulation in the blood stream. The transmission electron micrograph (TEM) clearly depicts the well-regulated particle size and high Hb content within the vesicles.

conducted a longer period of observation (two weeks) after moderate and clinically relevant isovolemic exchange transfusion of a 40% estimated blood volume with HbV suspended in a 5-g/dl rHSA solution [12]. All rats survived and increased their body weights until their intended sacrifice. We undertook plasma biochemical, hematological and histopathological examinations, which showed no irreversible changes.

The ability of HbV to restore systemic conditions after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6 h after resuscitation [13]. The HbV was suspended in a 5-g/dl rHSA solution (HbV/rHSA) at an Hb concentration of 8.6 g/dl. Shock was induced by a 50% blood withdrawal. The rats showed hypotension and considerable metabolic acidosis and hyperventilation. After 15 min, they received HbV/rHSA, shed autologous blood (SAB), washed homologous red blood cells (wRBC) suspended in rHSA (wRBC/rHSA, [Hb] = 8.6 g/dl), or rHSA alone. The HbV/rHSA group restored mean arterial pressure to 93 ± 8 mm Hg at 1 h, similar to the SAB group (92 ± 9 mm Hg), which was markedly higher than that of the rHSA group (74 ± 9 mm Hg). No remarkable difference in the blood gas variables pertained between the resuscitated groups. However, two of eight rats in the rHSA group died before 6 h. After 6 h, the rHSA group showed considerable ischemic changes in the right cerebral hemisphere relating to the ligation of the right carotid artery followed by cannulation, whereas the HbV/rHSA, SAB, and wRBC/rHSA groups showed less change. These results indicate that HbV suspended in albumin provides restoration from hemorrhagic shock that is comparable to that using shed autologous blood [13,14]. We are now studying a similar resuscitation study using beagles, confirming the effectiveness of HbV as a resuscitative fluid.

One characteristic of HbV is that the oxygen affinity of HbV can be regulated by changing the amount of co-encapsulated allosteric effector without changing other physicochemical properties. This provides a unique opportunity to explore new clinical indications: HbV with a high O_2 affinity (low P_{50}) can retain O_2 at an upper flow in the micro circulation and release O_2 at a hypoxic tissue where the blood flow is significantly reduced [15,16]. Actually, HbV with a high O_2 affinity improved oxygenation of an ischemic skin flap [17,18]; the results imply the possibility of further application of HbV to other ischemic diseases such as myocardial and brain infarction and stroke.

3. IN VIVO SAFETY OF HBV

We examined the safety profile of HbV with regard to microvascular responses, pharmacokinetics, influence on RES, influence on clinical measurements, and daily repeated infusion.

We observed the responses to the infusion of intra-molecularly cross-linked Hb (XLHb) and HbV into conscious hamsters. The XLHb (7 nm diameter) showed a significant increase in hypertension equal to 35 mmHg with simultaneous resistance caused by vasoconstriction of the artery equal to 75% of the baseline levels [19]. On the other hand, HbV at 250 nm showed minimal change. The small acellular XLHb is dispersed homogeneously in the plasma; it diffuses through the endothelium layer of the vascular wall and reaches the smooth muscle. The XLHb presumably traps nitric oxide (NO) as an endothelium-derived relaxation factor. Subsequently, it induces vasoconstriction and hypertension. On the other hand, the large HbV should stay in the lumen and does not induce vasoconstriction. Several mechanisms are proposed for Hb-induced vasoconstriction. These include NO-binding, excess O_2 supply, reduced shear stress, and the presence of a Hb recognition site on the endothelium. However, it is apparent that Hb-encapsulation shields the side effects of acellular Hbs.

The vascular wall of the sinusoid in hepatic microcirculation has many pores, called fenestrations, of ca. 100 nm diameter. The small Hb molecules with a diameter of only 7 nm extravasate through the fenestrated endothelium and reach the space of Disse. On the other hand, HbV particles, which are larger than the pores, do not extravasate. The heme of extravasated Hb is metabolized excessively by hemeoxygenase-2 in hepatocyte to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb binds CO rapidly, engendering vasoconstriction and increased vascular resistance. On the other hand, HbV (250 nm diameter) is sufficiently large to remain in the sinusoid; the vascular resistance is maintained [20].

These observations indicate the importance of cellular structure and particle diameter of HbV from the viewpoint of hemodynamics. However, the particles in this size range (250 nm) finally distribute to so-called reticuloendothelial system. Circulation persistence was measured monitoring the concentration of radioisotope-labeled HbV [21]. The circulation half-life is dose-dependent; it was 32 h in rats when the dose rate was 14 ml/kg. Gamma camera images showed the distribution of HbV mainly in the liver, spleen and bone marrow. Transmission electron microscopy (TEM) showed that the spleen 1 day after infusion of HbV demonstrated the presence of HbV particles in the phagosomes of the macrophages [22]. However, the HbV structure cannot be observed after 7 days. A pathological technique using anti-human Hb antibody showed the clearance of human Hb derived from HbV in one week.

One issue of the Hb-based O_2 carriers is that they have a marked influence on clinical laboratory tests. They remain in the plasma phase in hematocrit capillaries after centrifugation of blood samples and

interfere with colorimetric and turbidimetric measurements. In contrast, HbV is easily removable from blood plasma, either by ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran to enhance precipitation of HbV. A clear supernatant is obtained for accurate analyses [23]. This is an advantage of HbV over acellular Hb solutions. Accordingly, we examined the influence on organ functions by serum clinical laboratory tests after bolus infusion and daily repeated infusions of HbV [24,25]. Albumin, ALT, AST, and LDH, which reflect the liver function, were within normal ranges. Concentrations of bilirubin and ferric ion were maintained at a low level. The lipid concentration changed transiently, but returned to its original level in 2 wk. These results indicate that the membrane components of HbV, once they reappear from RES, are metabolized on the physiological pathway.

4. SUMMARY

Results showing the safety and efficacy of HbV have encouraged us to move forward. The joint collaboration partnership of academia (Waseda and Keio Universities), a biotech venture company (Oxygenix, Co. Ltd., Tokyo) and one corporation (Nipro Corp., Osaka) are striving to achieve clinical trials of HbV within a few years. Considerable efforts have been undertaken to produce HbV with a facility of GMP standard, and to start preclinical and, ultimately, clinical trials.

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—【論文記事】— Secondary Publication —

ヘモグロビン小胞体（HbV）-リコンビナントアルブミン分散溶液による40%交換輸血：ラット脾臓内HbV代謝と造血に関する2週間の観察†

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（背景）輸血代替として開発されたヘモグロビン小胞体（HbV，粒径251±81nm）について，動物投与試験によりその酸素運搬機能が明らかにされてきた．しかし投与後の中長期的な回復過程，特に細網内皮系におけるHbVの分解と造血については十分な検討が為されていなかった．（方法）Wistar系ラット（♂，60匹）を用い，HbVを5g/dL濃度のリコンビナントアルブミン溶液（rHSA）に分散させた溶液（HbV/rHSA），ラット保存赤血球をrHSAに分散させた溶液（sRBC/rHSA），或はrHSA溶液単独で，循環血液量の40%を急速交換した．その後最長14日間の血液学的，血液生化学的解析，および組織病理学的検討を実施した．（結果）HbV/rHSA群とrHSA群では，血液交換後に低下したヘマトクリット値（Hct，約26%）が，7日後には交換前の値（43%）にまで回復した．血中エリスロポエチン濃度は，全群で上昇した．特にrHSA群で1日後に最も高い値を示し（321±123IU/L），貧血状態を反映したと考えられた（HbV/rHSA，153±22；sRBC/rHSA，63±7；baseline，21±3）．また，同時に全群で脾臓肥大を認めた（HbV/rHSA＞rHSA＞sRBC/rHSA）．組織病理学的観察から，脾臓に捕捉されたHbVは14日以内に完全に消失した．しかし，ヘモジデリン沈着がHbV/rHSA群およびsRBC/rHSA群に認められた．また，rHSA群とHbV/rHSA群の赤脾髄に多量の赤芽球が存在した．（結論）40%交換輸血において，脆弱な赤血球が細網内皮系で捕捉分解される生理的機序と同様の経路を経て，HbVが捕捉代謝される過程が予想された．また，造血機能の亢進によりHctが7日以内に完全に支障無く回復することを確認した．

キーワード：人工血液，人工酸素運搬体，人工赤血球，リポソーム，代用血漿剤

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第53回日本輸血学会総会推薦論文

1. 緒　言

ヘモグロビン（Hb）を利用した人工酸素運搬体（Hb-based O_2 carriers，HBOCs）の研究が世界的規模で進展し，臨床試験の段階にある製剤も幾つか知られている[1][2]．HBOCの利点は，血液型が無いこと，病原体を完全に排除できること，また，赤血球に比較して長期間の保存が可能なことである[3]．血中滞留時間が赤血球に比較して2～3日と短いものの[4]，様々な短時間の用途が期待されている．例えば，1）緊急時の出血性ショックの蘇生液として，輸血までの繋ぎとしての投与[5]，2）術前の血液希釈，術中の出血に対する投与により，輸血の回避，或は輸血を遅らせる手段[6]，3）心臓手術の際に使用する体外循環回路の補充液としての利用，4）酸素治療剤として，虚血性疾患など局所的低酸素状態の改善薬としての利用，などがある[8][9]．

筆者らが開発したヘモグロビン小胞体（HbV）は，

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〔受付日：2006年10月5日，受理日：2006年12月12日〕

期限切れ赤血球から精製した高純度高濃度ヒトHb溶液を脂質膜で被覆した微粒子構造をしている[10][11]．赤血球と類似の細胞構造により，本来毒性を有する分子状Hbと血管内皮細胞との直接的接触の回避が可能となる[12][13]．血中に投与されたHbVは，酸素運搬機能を終えた後，最終的に細網内皮系(RES, Reticuloendothelial system)マクロファージに捕捉，分解されることが負荷投与試験から明らかになっている[14]~[17]．これまでに，HbVをヒト血漿由来のアルブミン或はリコンビナントアルブミン溶液(rHSA)に分散させ，循環血液量の80～90%を交換する極度の血液希釈試験，50%脱血による出血性ショックモデルに対する蘇生試験等で，急性期において赤血球と同等の酸素運搬機能が実証されてきた[18]~[21]．しかし，投与後数時間の観察に留まっており，中長期的な観察は充分になされていなかった．

そこで本研究では，HbVをrHSA溶液に分散させた溶液を用い，臨床的に想定される40%の血液交換をラットモデルを用いて行い，その後2週間に亘る観察を行った．血液生化学検査，血液学的検査，組織病理学的検査を行い，特にRESにおけるHbVの代謝とHctの回復過程について注目した．単回負荷投与，或は反復負荷投与の際には，肝臓よりも脾臓の肥大が顕著であり[14][15][17]，更に，老化赤血球は脾臓にて捕捉代謝されることが知られているので[22]，本研究では，輸血モデルとして保存赤血球を投与した場合も実施し，脾臓への影響を比較検討した．

2. 材料・方法

2-1. rHSAに分散させたHbVの調製

HbVは無菌的条件にて，既報に従って調製した[23][24]．精製ヒトHb溶液は，日本赤十字社から提供を受けた期限切れヒト赤血球より調製した．HbVは38g/dLのHb溶液を内包している．アロステリック因子としてpyridoxal 5'-phosphate (PLP)を含有する(Sigma-Aldrich Co., St. Louis, MO, PLP：Hb＝2.5：1 by mol)．HbVの脂質膜には，1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate(日本精化，大阪)，および1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}(日本油脂，東京)が，5/5/1/0.033の割合で存在している．エンドトキシン含量は，リムルス法の変法により0.1EU/mL以下であることを確認した[25]．物理化学的パラメータとして，酸素親和度(P_{50})が27Torr，粒径251±81 nm，metHb含量は3%以下であった．HbV分散液([Hb]＝10g/dL) 8.6mLをrHSA溶液(25g/dL) 1.4 mLと混合し，HbVが分散している外液のrHSA濃度を5g/dLに調節した．従って，得られたHbV/rHSAのHb濃度は8.6g/dL |＝10g/dL×8.6/(8.6＋1.4)| になる．このとき膠質浸透圧は20Torr，粘度2.9cPになる．

2-2. rHSAに分散させたラット保存血の調製

エーテル麻酔下，Wistar系ラットの下大静脈から急速脱血した．ラット血液10mLに対し，血液保存液(CPDA-1，川澄化学社製)を1mL加え，密封して4℃にて1週間冷蔵保存した．報告によれば，1週間保存したラット赤血球の脆弱性は，ヒト赤血球を同条件で4週間保存したものと同等になる[26]．保存後，遠心分離(4,000 g，10min)して血清と白血球を除去し，再度生理食塩水を等量加えて遠心分離する操作を二回繰り返した．次いで5g/dLのrHSAを添加して再分散させ，再度遠心分離して上澄みを除去し，rHSAを添加してHb濃度を8.6g/dLに調節した(sRBC/rHSA)．

2-3. 血液交換試験と2週間の観察

Wistar系ラット65匹を用いた(♂，223±20g)．ネンブタール(Abbot Laboratories, North Chicago, IL)を腹腔内投与し(1mL/kg)麻酔状態とし，頸動脈にポリエチレン製カテーテル(SP31)を挿入した．1mL/30 secの脱血と同量の試料溶液投与を繰り返して40%の血液を交換した．試料溶液は，HbV/rHSA(HbV群，n＝20)，sRBC/rHSA(sRBC群，n＝20)，およびrHSA単独の投与(n＝20)である．ベースラーンの値を知るため，5匹のラットを使用した．

循環血液量は56mL/kg体重と推定し[27]，これを維持しながら血液を交換すると仮定し，実験に必要なHbV分散液の量を計算した．1.0mLの脱血と試料等投与を繰り返したと仮定すると，40%の血液交換は次式で示される．

$$40\% = 100 \times |1 - [(0.056 \times 体重 - 1.0)/(0.056 \times 体重)]^{n}| \quad (1)$$

総投与量は，$n \times 1.0$(mL)と計算できる[28]．体重220 gのラットの場合，必要量は6.0mLになる．

血液交換終了後，カテーテルを外し，右頸動脈を結紮，切開部を縫合した．ラットはその後，ケージに入れ最長14日間生存させた．投与後1，3，7，14日目に各群から5匹を選択し，1.5%セボフルレン吸入麻酔下，尾静脈より24G-留置針(ニプロ社製)を使用して採血(150μL)し，ヘマトクリットと血球数測定を行った(Model KX-21，シスメックス，神戸)．開腹して下大静脈から採血し，血液生化学検査の検体とした．その後直ちに臓器を摘出し，10%ホルマリン中性リン酸溶液に浸漬固定し，パラフィン包埋した．4μm厚の切片について，ヘマトキシリン-エオジン(H/E)染色，ベルリンブルー染色，および，ギムザ染色を実施した．

脱血液(約6mL)を遠心分離(5,000g，10分)し，血漿を得た．HbVが血漿中に残存している場合(1，3日後)には，更に超遠心分離(50,000g，20分)によりこれを沈降分離し，透明な血漿層を測定検体とし，血

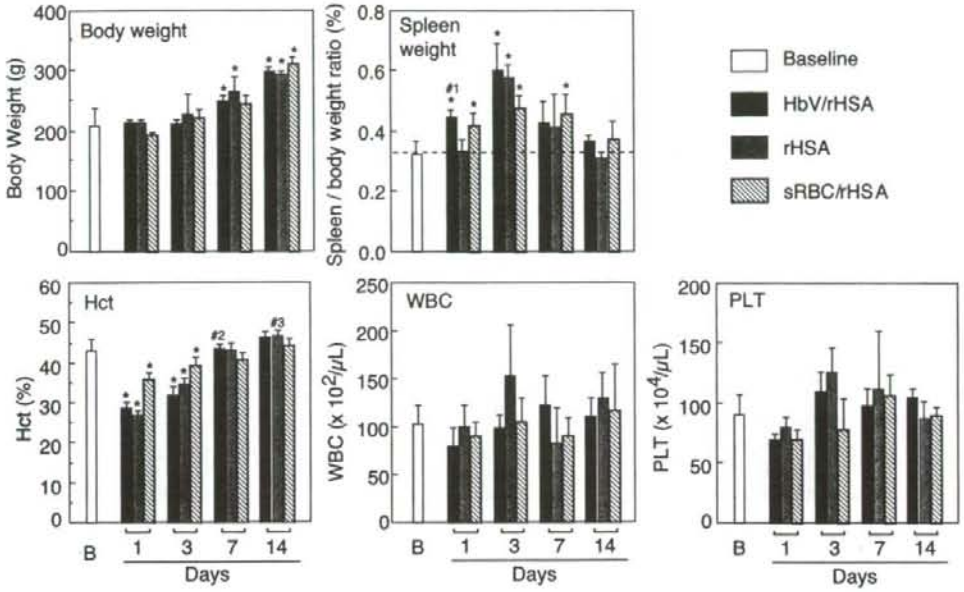

図1　40% 血液交換後の体重，脾臓重量比，および血球数の変化．$*p < 0.01$ vs baseline；$^{\#1}p < 0.01$ vs. rHSA 群；$^{\#2}p = 0.0288$ vs. sRBC/rHSA；$^{\#3}p = 0.0353$ vs. sRBC/rHSA. B：baseline.（From：Sakai et al., Transfusion 2006；46：339－347, Blackwell Publishing, Oxford, UK）

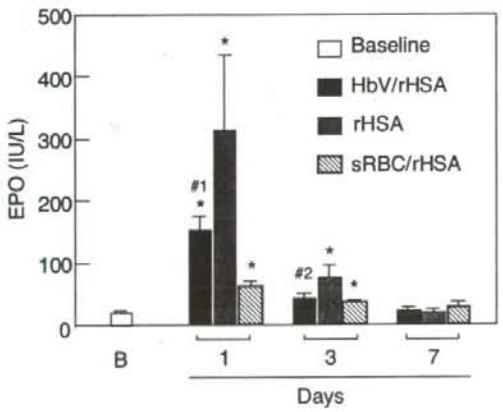

図2　40% 血液交換後の血中エリスロポエチンの濃度変化．$*p < 0.01$ vs baseline；$^{\#1}p = 0.0222$ vs. rHSA；$^{\#2}p = 0.0195$ vs. rHSA. B：baseline.（From：Sakai et al., Transfusion 2006；46：339－347, Blackwell Publishing, Oxford, UK）

液生化学検査における HbV の干渉作用を排除した[29]．得られた透明な血漿には Hb が含まれず，HbV の溶血が無いことを示した．血漿検体は検査まで－80℃ にて凍結保存した．測定項目は，肝臓，腎臓機能等を反映する通常の検査項目のほか，エリスロポエチン（EPO），および HbV の分解排泄を反映すると考えられる脂質成分と，ビリルビン，遊離鉄等とした（BML 社）．ラットの EPO は，ヒトの EPO との相同性が高いので，抗ヒト EPO 抗体での検出が可能であった[30]．

動物実験は慶應義塾大学医学部動物実験委員会の承認を得て実施した．また，Guide for the Care and Use of Laboratory Animal の指針に従った[31]．

2-4. 統計処理

結果は全て平均±標準偏差（SD）として記した．コントロール群と処置群の間の有意差検定には，Fisher's protected least significance difference test および one way ANOVA 法を用いた．p 値が 0.01 以下のときに有意な差と判定した．

3. 結　果

3-1. 体重，脾臓重量，血液学的検査の結果

全群が 40% 交換輸血に耐え，犠牲死させるまで生存した．これは，5g/dL の rHSA 溶液の使用により，膠質浸透圧と循環血液量を一定に保ち乍ら血液希釈を行ったことが理由として先ず考えられる．体重（約 223g）は 14 日後には 300g 程度にまで成長した（図 1）．立毛などの異常な症状は認められなかった．

体重に対する脾臓重量の変化では，HbV/rHSA 群で特に，1，3 日後に有意に増大していたが，14 日後には正常値に復した．rHSA 群では 1 日後には変化は無いが，3 日後に急激に増大し，その後低下して 14 日後には正常値に復した．sRBC 群は HbV 群よりは程度は低いものの 1 日後から 7 日後まで脾臓肥大が見られた．

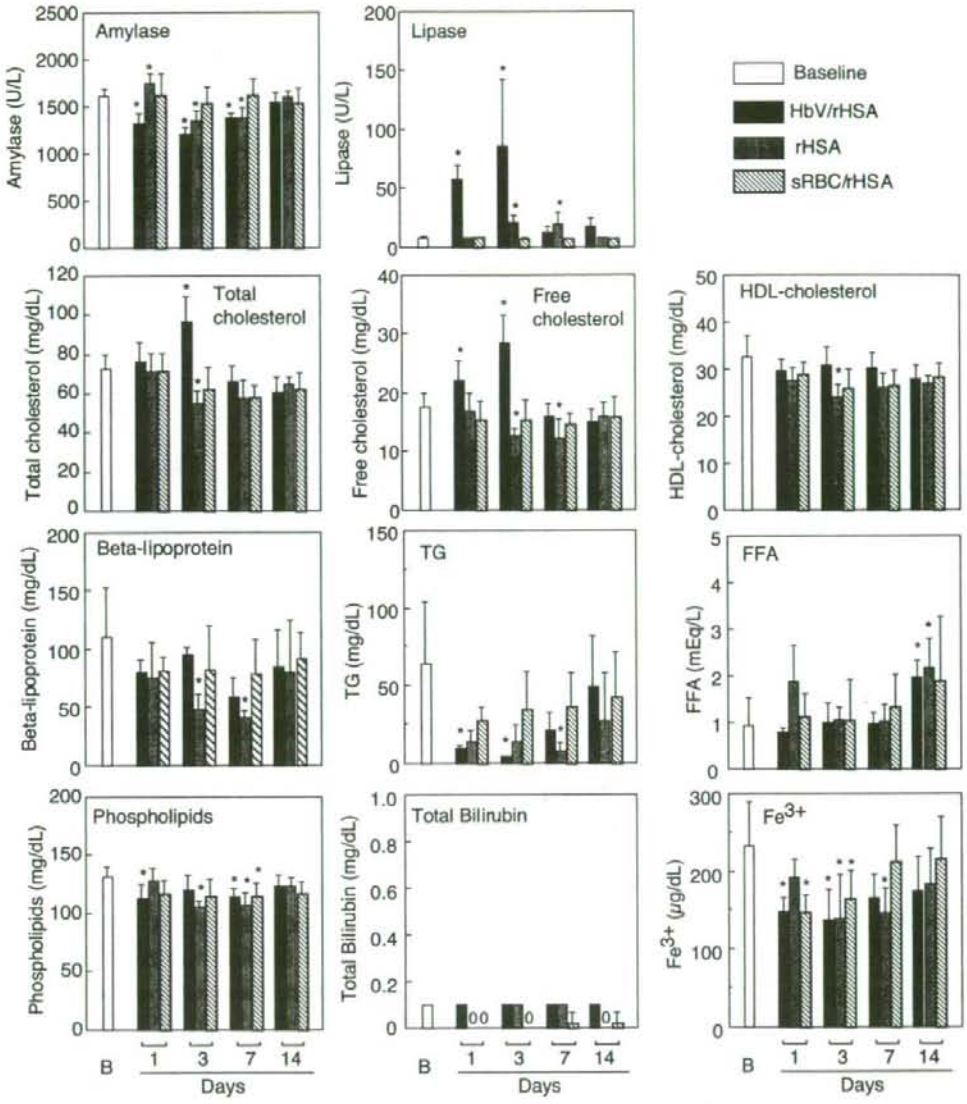

図3　40% 血液交換後の血液生化学検査の結果．* $p < 0.01$ vs. baseline. Triglyceride, TG；free fatty acid, FFA；baseline, B.（From：Sakai et al., Transfusion 2006；46：339－347, Blackwell Publishing, Oxford, UK）

Hct 値は，血液交換前は 43% であったが，HbV/rHSA 群および rHSA 群は，血液交換後 Hct 値が 26% に低下した．7 日後には交換前の値（43%）に復し，更に 14 日後には 46% になり，交換前の値を超えた．sRBC/rHSA 群では，投与後の値は高いが，7，14 日後では他の二群に比較して低めの値を示した．平均赤血球ヘモグロビン量（MCH），平均赤血球容積（MCV），平均赤血球ヘモグロビン濃度（MCHC）に異常を認めなかった．但し，HbV/rHSA 群では，1，3 日後に血液中に HbV が残存しているため，測定不能であった．sRBC/rHSA 群では，1 日後の MCH および MCHC で低下傾向がみられた．Hct 値とは対照的に，血小板数および白血球数は，安定した値を推移した．HbV 由来の血漿中 Hb 濃度は，血液交換直後は 4.4g/dL と想定され，その後，1，3，7 日後に 1.8±0.1，1.1±0.1，0g/dL に低下した．

3-2. 血液生化学検査

血中 EPO 値は，貧血，低酸素状態，ストレス等を反映する項目であり，血液交換前の 21±3IU/L が，1 日後に rHSA 群が 321±123IU/L を示し，これは HbV/rHSA 群（153±22），sRBC/rHSA 群（63±7）よりも高値であった（図 2）．しかし 3 日目には低下傾向にあり，7 日後には処置前の値に復した．

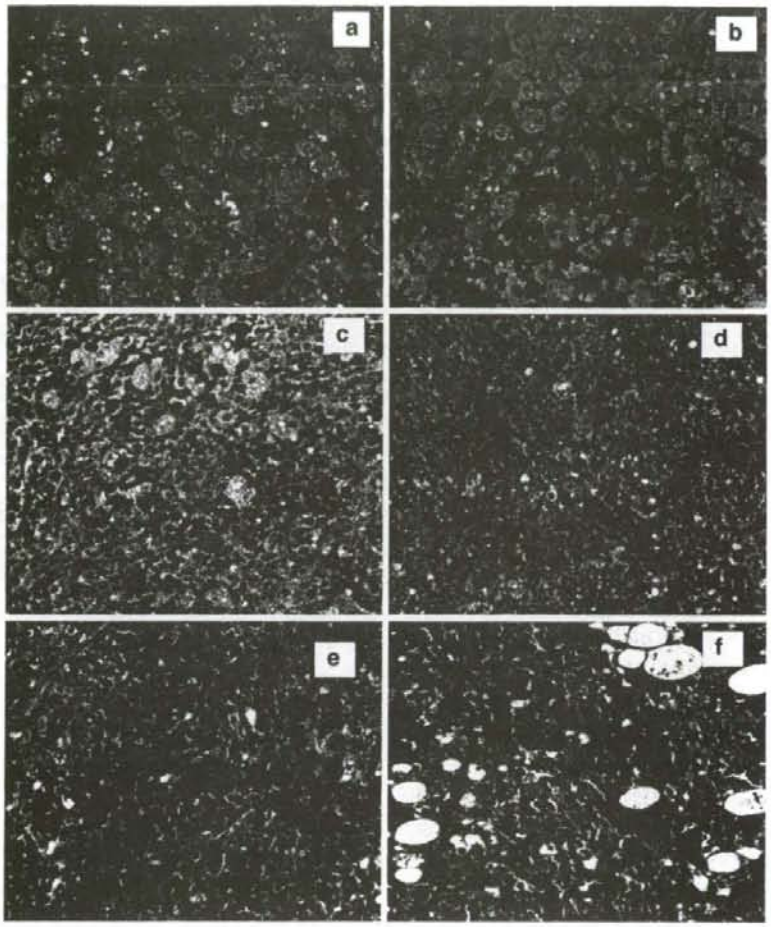

図4　HbV/rHSA群およびrHSA群の組織切片のギムザ染色像．(a)，(b)，(c)は，HbV/rHSA群の1，3，7日後の脾臓．捕捉されたHbVは薄青色の領域（黒矢印）．赤芽球巣は濃青色の細胞として確認される（赤矢印）．HbVは7日後には可成り減少する．(d) HbV/rHSA群の14日後の脾臓．HbVは完全に消失した．(e) rHSA群の3日目の脾臓．赤芽球巣が多く認められる．(f)HbV/rHSA群の3日目の骨髄にも濃青色の赤芽球が存在する．紫のスポットが点在する細胞は好塩基球と考えられる．スケールバーは50μm．（From：Sakai et al, Transfusion 2006；46：339－347, Blackwell Publishing, Oxford, UK）

その他，AST値が1日目に若干の上昇傾向を示したが（HbV/rHSA，70±5U/L；rHSA，69±12；sRBC/rHSA，72±9；正常値，60±7），3日後には正常値に復した．ALT値は特に変化は無かった．ALPおよびγ-GTPには多少の変動があった．CPKは安定していた．全群について，クレアチニン，尿酸は低値傾向を示した．アミラーゼに低下傾向が見られたが(図3)，HbV/rHSA群のみ，リパーゼの上昇を認めたが，7日後には正常値に復した．脂質成分濃度については，HbV/rHSA群で血中の総コレステロール，遊離コレステロールの亢進が3日後に見られたが，7日後には正常値に復した．β-リポ蛋白，高密度リポ蛋白コレステロールは，血液交換後に特にrHSA群で低下傾向があった．トリグリセリドも全群で低下傾向にあり，特にHbV/rHSA群で1，3日後に顕著であった．これは，血漿を超遠心分離した際に分離されたことも一因と考えられた．リン脂質は全群で僅かに低下傾向にあった．遊離脂肪酸は14日後に増加傾向にあった．ビリルビン濃度(<0.1mg/dL)は常に低値を推移した．遊離鉄濃度は低下傾向にあったが，14日後には血液交換前の値に復した．

3-3. 組織病理学的検討

全群において肺，心臓，腎臓等に著変を認めなかった．HbV/rHSA群では1，3日後に肝臓のクッパー細胞，骨髄のマクロファージに捕捉されたHbVが観察された