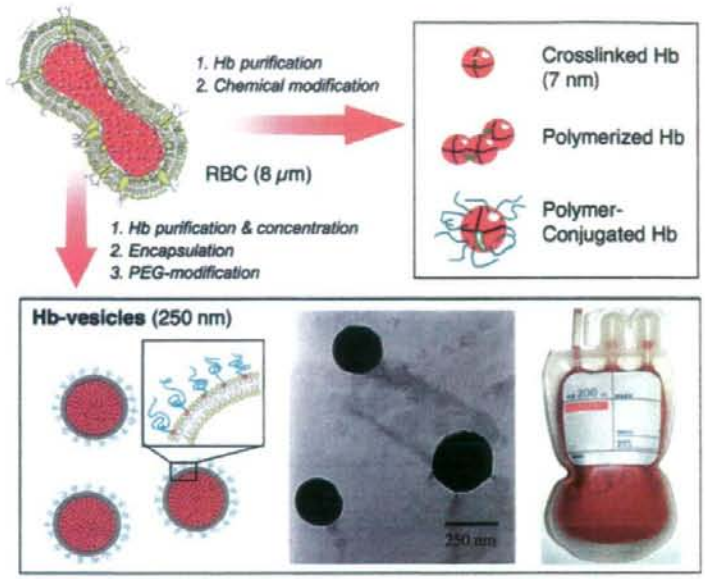


**Figure 1.** Schematic representation of a series of Hb-based oxygen carriers. In the case of Hb-vesicles (Hbv), the purified and concentrated Hb solution (35 g/dL) is encapsulated in phospholipid vesicles and the surface is modified using PEG chains. The particle size is well regulated at 250 nm, which is apparent from the transmission electron micrograph. The color of HbV is like a mixture of red wine and milk, due to the light scattering effect of small particles. Hb, hemoglobin; PEG, polyethyleneglycol; RBC, red blood cell.

cells, such as the concentration of phosphates [2,3-diphosphoglycerate (2,3-DPG), adenosine triphosphate, etc.] and other electrolytes; (v) RBCs are the major component that renders blood as non-Newtonian and viscous, which is necessary to pressurize the peripheral artery for homogeneous blood distribution and for maintenance of blood circulation[10]; and (vi) the cellular structure of RBCs retards oxygen release in comparison to acellular Hb solutions,[11,12] thereby retaining oxygen to peripheral tissues where oxygen is required.

For those reasons, the optimal structure of HBOCs might be to mimic the RBC cellular structure. The pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed in 1957 by Chang,[13] who prepared microcapsules (5 μm) made of nylon, collodion and other materials. Toyoda[14] in 1965 and the Kambara-Kimoto group[15] in 1968 also investigated encapsulation of Hbs with gelatin, gum arabic, silicone, etc. Nevertheless, results emphasized the extreme difficulty in regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne[16] reported in 1964 that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller[17] in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. The US Naval Research Laboratories showed remarkable progress in the use of LEH.[18–20] Terumo Corporation (Tokyo) developed a different LEH called Neo Red Cells (Table 1).[21,22]

However, some intrinsic issues of encapsulated Hbs remained, which were mainly related to the nature of molecular assembly and particle dispersion. What we call HbV, with their high-efficiency production processes and improved properties, were established by our group based on technologies of molecular assembly in concert with precise analyses of pharmacological and physiological aspects (Table 2).[23–25] We use stable carbonylhemoglobin (HbCO) for purification with pasteurization at 60°C for 10 hours. The purity of the obtained Hb solution is extremely high.[26,27] Utilization of the stable and purified HbCO enables higher concentrations than 40 g/dL using ultrafiltration and easy handling of encapsulation by the extrusion method without causing protein denaturation. It has been confirmed that HbV encapsulates nearly 35 g/dL with a thin bilayer membrane. In the final process, HbCO in HbV is photodissociated by irradiation of visible light under an oxygen atmosphere to convert $HbO_2$.[28]

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**Table 1. A list of representative LEH extensively studied aiming at industrialization**

| Product Name | Group | Characteristics | Current status |
|---|---|---|---|
| Hb-vesicles (HbV) | Waseda University and Keio University | 1. Pasteurization of HbCO at 60°C for virus inactivation, and high purity and concentration of encapsulated Hb<br>2. Lipid composition to improve blood compatibility<br>3. PEG modification and deoxygenation for 2 years storage<br>4. (Hb) = 10 g/dL | Preclinical |
| Neo Red Cells (NRC) | Terumo Corporation | 1. Inositol hexaphosphate to regulate $P_{50}$ (= 40–50 torr)<br>2. Lipids: HSPC/cholesterol/fatty acid/PEG-lipid<br>3. Storage in a refrigerator for 6 months<br>4. (Hb) = 6 g/dL | Preclinical |
| Artificial Red Cells (ARC) | NOF Corporation and Waseda University | 1. Polymerized lipids (DODPC) for stabilization<br>2. Storage in powdered or frozen state<br>3. Difficulty in degradation in RES | Suspended |
| LEH | US Naval Research Laboratory | 1. Freeze-dried powder with trehalose<br>2. Low Hb encapsulation efficiency<br>3. Thrombocytopenia, complement activation | Suspended |
| Synthetic erythrocytes | Rush-Presbyterian-St. Luke's Medical Center, University Illinois | 1. The first attempt of LEH | Suspended |

DODPC, 1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; Hb, hemoglobin; HbCO, carbonylhemoglobin; HSPC, hydrogenated soy phosphatidylcholine; LEH, liposome-encapsulated Hbs.

**Table 2. Characteristics of Hb-vesicles developed in Waseda University**

| Parameter | |
|---|---|
| Particle diameter | 240–280 nm |
| $P_{50}$ | 25–28 torr |
| (Hb) | 10 g/dL |
| Suspending medium | Physiologic saline solution (0.9% NaCl) |
| Colloid osmotic pressure | 0 torr |
| Intracellular Hb concentration | ca. 35 g/dL |
| Lipid composition | DPPC/cholesterol/DHSG/DSPE-$PEG_{5000}$ |
| Weight ratio of Hb to lipids | 1.6–1.9 (w/w) |
| Stability for storage at room temperature | 2 years |
| Circulation half-life | 32 hours (rats) |

DHSG, 1,5-*O*-dihexadecyl-N-succinyl-L-glutamate; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidyethanolamine-*N*-polyethyleneglycol; Hb, hemoglobin.

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The oxygen-bound HbV can provide oxygen-transport capacity that is both sufficient and comparable to that of RBCs in experiments related to extreme blood exchange[29-34] and fluid resuscitation from hemorrhagic shock.[35-38] A recent experiment of HbV as a priming solution for cardiopulmonary bypass in a rat model showed that HbV protects neurocognitive function by transporting oxygen to brain tissue even when the hematocrit is markedly reduced.[39] Other studies investigating HbV suspension as a possible perfusate for organ transplantation are also underway for the heart, liver, intestine, etc.

In fact, Hb encapsulation provides a unique opportunity to add new functions to particles. Other regulators, such as antioxidants and enzymes, can be embedded on the capsule or coencapsulated to reduce methemoglobin (metHb),[40-42] as can allosteric effectors to modulate oxygen affinity ($P_{50}$).[13,43] The $P_{50}$ of HbV is regulated by coencapsulation of pyridoxal 5′-phosphate (PLP) in place of 2,3-DPG. The present HbV, being developed by Waseda University, contains PLP at PLP/Hb = 2.5 by mol; the resulting $P_{50}$ is about 25–28 torr, which shows sufficient oxygen transporting capacity as a transfusion alternative. The $P_{50}$ of HbV without PLP and $Cl^-$ is 8–9 torr. This formulation is effective for targeted oxygen delivery to anoxic tissues caused by reduced blood flow.[14,44,45]

In addition to HbV, new encapsulated Hbs without liposomes have emerged with the use of recent advanced nanotechnologies, such as polymersome,[46] polyethyleneglycol (PEG)-poly(ε-caprolactone) copolymer nanoparticles,[47] and *in vivo* evaluation of oxygen-carrying capacities of these new materials is anticipated. Encapsulation of Hb can reduce the toxicity of cell-free Hbs. However, many hurdles must be surmounted to realize encapsulated Hbs because of the components of the capsules themselves and their structural complexity as a molecular assembly. It is also important to consider the larger dosage requirement of encapsulated Hb for blood substitution in comparison with those available with conventional drug delivery systems, which require no large dosage.

## STRUCTURAL STABILIZATION OF ENCAPSULATED HEMOGLOBIN FOR STOCKPILING

Hb autoxidizes to form metHb and loses its oxygen-binding ability during storage, as well as during blood circulation. Therefore, prevention of metHb formation is necessary. A method exists to preserve deoxygenated Hbs in a liquid state using well-known intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the oxygen partial pressure; also, deoxyHb is not autoxidized at ambient temperatures.[48] In the case of HbV, not only the encapsulated Hb but also the capsular structure (liposome) must be physically stabilized to prevent irreversible intervesicular aggregation, fusion and leakage of the encapsulated Hb.

Liposomes, as molecular assemblies, have been generally inferred to be structurally unstable. The US Naval Research Laboratory tested the addition of cryoprotectants and lyoprotectants, such as trehalose, to LEH for its preservation as a powder without causing hemolysis after rehydration.[49,50] In addition, many researchers have developed stabilization methods for liposomes that use polymer chains.[51-54] Polymerization of phospholipids that contain two dienoyl groups (DODPC) was studied extensively in our group. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in DODPC. This method produces enormously stable liposomes, resembling rubber balls, which are resistant to freeze-thawing, freeze-drying and rehydration.[55,56] However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages, even 30 days after injection.[57] It became widely believed that polymerized lipids are inappropriate for intravenous injection. Subsequently, it was clarified that the selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition are important to enhance the stability of nonpolymerized liposomes.[31,58] Surface modification of liposomes with PEG chains is sufficient for dispersion stability.[32] In fact, in comparison to RBCs, HbV is highly resistant to hypotonic shock, freeze-thawing and enzymatic attack by phospholipase $A_2$.

We investigated the possibility of long-term preservation of HbV during storage for 2 years through a combination of two techniques: deoxygenation and PEG modification.[59] The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion for 2 years because of their steric hindrance.[60] The original metHb content (ca. 3%) before preservation decreased gradually to less than 1% in all samples after 1 month because of the presence of a

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reductant, such as homocysteine, inside the vesicles that consumed the residual oxygen and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the oxygen partial pressure: a lack of increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for 2 years. These results indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency.

## BLOOD COMPATIBILITY OF LIPOSOMES AND HEMOGLOBIN VESICLES

Liposome is not a solute but a particle in a suspension. The surface of the particle may be recognized, interact with blood components, including complements. The so-called *injection reaction*, or pseudoallergy, is caused by complement activation, giving rise to anaphylatoxins that trigger various hypersensitivity reactions. This reaction is sometimes observed not only with liposomal products,[61] but also with fat emulsions[62] and a perfluorocarbon emulsion.[63] Therefore, the examination of blood compatibility of encapsulated Hbs is important for clinical use. Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol) developed by the US Naval Research Laboratory[64,65] and of other products. In our group, exchange transfusion with the proto-type HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension.[30] Similar effects were also observed for administration of negatively charged liposomes.[66,67] The transient reduction in platelet counts caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver. Such nonphysiological platelet activation probably leads to initiation and modulation of inflammatory responses as platelets contain an array of potent proinflammatory substances. However, it must be emphasized that the present HbV formulation apparently does not induce thrombocytopenia in animal experiments, probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate), not DPPG or a fatty acid.[68-70]

Detailed blood compatibility of HbV in relation to negatively charged lipid was examined by Dr H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo) and his colleagues.[69-72] The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was activated slightly by the proto-type DPPG-HbV, but not by the present PEG-DHSG-HbV. Moreover, the complement consumption in the plasma was detected by incubation with DPPG-HbV, but not with the present PEG-DHSG-HbV.[71] The exposure of human platelets to high concentrations of the present HbV (up to 40%) *in vitro* did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists. These results imply that HbV, at concentrations of up to 40%, has no aberrant interactions with either unstimulated or agonist-induced platelets. It can be concluded that the present PEG-DHSG-HbV has a higher blood compatibility.

## BIODISTRIBUTION, METABOLISM AND EXCRETION OF HEMOGLOBIN VESICLES

The dosage of blood substitutes should be considerably larger than those of other drugs, while their circulation time is considerably shorter than that of RBCs. Therefore, their biodistribution, metabolism, excretion and side effects must be characterized in detail, especially in relation to the reticuloendothelial system, RES (or termed the mononuclear phagocytic system).

Normally, free Hb released from RBC is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Hemoglobinuria and eventual renal failure occur when the reabsorption capacity of the kidney is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES in much the same manner as senescent RBCs are, as confirmed by radioisotope $^{99m}$Tc-labelled HbV

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injection.[19,68] The HbV are finally distributed mainly in the liver, spleen and bone marrow. The circulation half-life is dose-dependent; when the dose rate was 14 mL/kg, the circulation half-life was 32 hours in rats. The circulation time in the case of the human body can be estimated as two or three times longer; or about 2 or 3 days at the same dose rate.

It is generally accepted that the liposome clearance by RES at a small dosage is accelerated by opsonization (absorption of plasma proteins such as antibodies and complements on the liposomal surface); PEG-modification prevents opsonization for prolonged circulation times.[73] However, considering the condition that the dosage of HbV is extremely high and requires a considerable amount of opsonins, and that HbV does not induce complement activation,[71] then the opsonin-dependent phagocytosis would not be a major component in the case of HbV with a large dosage. Actually, opsonin-independent phagocytosis, a direct recognition by macrophages, has been clarified in some studies.[74,75]

Transmission electron microscopic analysis of the spleen 1 day after infusion of HbV revealed the presence of HbV particles in the phagosomes of macrophages.[76] However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. Immunochemical staining with a polyclonal antihuman Hb antibody was used as the marker of Hb in the HbV, clarifying that HbV almost disappeared after 7 days in both the spleen and liver.

Bilirubin and iron are believed to be released during metabolism of Hb, but our animal experiments of topload infusion, daily repeated infusions and 40% blood exchange showed that neither of those products increased in the plasma within 14 days.[77-79] The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages. Bilirubin would normally be excreted in the bile as a normal pathway; no obstruction or stasis of the bile is expected to occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Hemosiderosis often occurs in patients who have received repeated blood transfusions because of the shorter half-life of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with lowered membrane deformability and shortened circulation half-life.[79]

As for membrane components of Hb-vesicles, phospholipids are metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and $CO_2$ in exhaled air. The plasma cholesterol level elevated transiently 3 days after injection, that was released from macrophages after degradation of HbV in the phagosomes.[77,79] However, the plasma phospholipid level did not increase significantly. It was recently clarified using $^3$H-cholesterol that the cholesterol of HbV is released from macrophages to blood; it is ultimately excreted in the feces. The PEG chain is widely used for surface modification of liposomal products. The chemical crosslinker of PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which are known as inert macromolecules, should be excreted in urine through the kidneys.[80]

In order to know the physiological capacity of RES for degradation of HbV, we tested massive intravenous doses by daily repeated infusion of 10 mL/kg/day into Wistar rats for 14 days. The cumulative dosage was 140 mL/kg (Hb and lipids, 20,689 mg/kg). The total volume was equal to 2.5 times of whole blood volume (56 mL/kg).[78] Even though the splenohepatomegaly was significant, all rats tolerated the infusions, and the body weight increased until the intentional sacrifice for the succeeding 14 days. The phagocyted HbV disappeared though significant hemosiderin deposition and was confirmed in the spleen, liver, kidney, adrenal grand and bone marrow. We could not define a lethal dose of HbV in this experiment.

The profile of liposome clearance is species-dependent. More precise data are necessary to extrapolate the phenomena observed in animal experiments to humans. However, these results imply that the metabolism and excretion of HbV are within the physiological capacity that has been well characterized for the metabolism of senescent RBCs and conventional liposomal products.

## UNIQUE RHEOLOGICAL PROPERTY OF HEMOGLOBIN VESICLES SUSPENSION

The extremely high concentration of the HbV suspension [(Hb) = 10 g/dL; (lipids) = 6 g/dL, volume fraction, ca. 40 vol%] imparts an oxygen-carrying capacity that is comparable to that of blood. The HbV suspension does

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not possess a colloid osmotic pressure (COP) because one HbV particle (ca. 250 nm diameter) contains about 30,000 Hb molecules, and HbV acts as a particle, not as a solute. Therefore, HbV must be suspended in or coinjected with an aqueous solution of a plasma substitute. This requirement is identical to that for emulsified perfluorocarbon, which does not possess COP;[81,82] it contrasts to characteristics of other HBOCs, intramolecular crosslinked Hbs, polymerized Hbs and polymer conjugated Hbs, which all possess very high COP as protein solutions.[8,83]

Animal tests of HbV suspended in plasma-derived human serum albumin (HSA) or recombinant HSA (rHSA) showed an oxygen-transporting capacity that is comparable to that of blood.[16,39] We reported previously that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian: no aggregation was detected microscopically.[31,32] In Japan, rHSA will be approved for clinical use in 2007,[84] but various plasma substitutes are used worldwide, such as hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG). The selection among these plasma substitutes should be determined not only according to their safety and efficacy, but also by the related price, experience of clinicians and customs of respective countries. Water-soluble polymers generally interact with particles such as polystyrene beads, liposomes and RBCs to induce aggregation or flocculation.[85,86] For that reason, it is important to determine the compatibility of HbV with these plasma substitutes. With that background, we studied rheological properties of HbV suspended in these plasma substitute solutions using a complex rheometer and a microchannel array.[87] The rheological property of an HBOC is important because the infusion amount should be considerably large, which might affect the blood viscosity and hemodynamics.

The HbV suspended in rHSA was nearly Newtonian. Its viscosity was similar to that of blood, and the mixtures with RBCs at various mixing ratios showed viscosities of 3–4 cP. Other polymers, HES, DEX and MFG, induced flocculation of HbV, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with the *shear-thinning* profile.[87] These HbV suspensions showed a high viscosity and a high storage modulus ($G'$) because of the presence of flocculated HbV. On the other hand, HbV suspended in rHSA exhibited a very low $G'$. The viscosities of HbV suspended in DEX, MFG and high molecular weight HES solutions responded quickly to rapid step changes of shear rates of 0.1–100 $s^{-1}$ and a return to 0.1 $s^{-1}$, indicating that flocculation formation is both rapid and reversible. Microscopically, the flow pattern of the flocculated HbV perfused through microchannels (4.5 μm deep, 7 μm wide, 20 $cmH_2O$ applied pressure) showed no plugging. Furthermore, the time required for passage was simply proportional to the viscosity.

It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower viscosity decreases shear stress on the vascular wall, causing vasoconstriction and reduced functional capillary density.[88] Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. The large molecular dimension of HbV result in a transfusion fluid with high viscosity. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors. These new concepts suggest reconsideration of the design of artificial oxygen carriers.[89] Actually, new products are appearing, although they are in the preclinical stage, not only HbV, but also zero-link polymerized Hb[90] and others with larger molecular dimensions and higher oxygen affinities.[91] Erni *et al.* clarified that HbV with a high $O_2$ affinity (low $P_{50}$, such as 8–15 torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischemic skin flap.[45,92,93] That study showed that HbV would retain $O_2$ in the upper arterioles, then perfuse through collateral arteries and deliver oxygen to the targeted ischemic tissues, a concept of targeted oxygen delivery by an HBOC.[44] Some plasma substitutes cause flocculation of HbV and hyperviscosity. However, reports show that hyperviscosity would not necessarily be deteriorative and might be, in some cases, advantageous in the body.[10] The combination of HbV and plasma substitute solutions provides a unique opportunity to manipulate the suspension rheology, not only as a transfusion alternative, but also for other clinical applications, such as oxygenation of ischemic tissues and *ex vivo* perfusion systems.

## CONCLUSION

Other related issues for HbV in a clinical situation include the interference effect of HbV on spectrophotometric measurements in routine clinical laboratory tests

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and noninvasive pulse oximetry monitoring of arterial blood oxygen saturation. Such interference is caused by strong light scattering resulting from the small HbV particles in blood.[94] We clarified that HbV can be removed easily from a blood specimen by the addition of high molecular weight dextran and centrifugation. Pulse oximetry can be improved by some modifications of the detection wavelength and software.

Encapsulation of Hb was initiated with the simple idea of duplicating the structure and function of RBCs. However, we are convinced that obstacles remain for the approach to realize the sophisticated function of RBCs; for example, it is impossible to mimic the flexibility of the unfilled biconcave structure of RBCs. The present HbV lacks ionophores in the bilayer membrane which facilitate the transport of small functional molecules from the outer medium, such as ascorbic acid or glutathione, to reduce metHb in HbV that does not contain enzymatic metHb reducing system, because the unstable enzymes are removed during the virus inactivation process of Hb purification.[26,27] On the other hand, clear advantages of simplified HBOCs exist, such as the absence of blood-type antigens and infectious viruses, along with stability for a long-term storage at room temperature for any emergency, all of which might overwhelm the functions of RBCs. The shorter half-life of the HBOCs in the bloodstream (2–3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties of HbV such as $P_{50}$ and viscosity supports the possible development of tailor-made oxygen carriers that suit various clinical indications. The achievements of ongoing HbV research described above give us confidence in advancing further development of HbV, with the expectation of its eventual realization.

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## DISCLOSURE

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# FLUID RESUSCITATION WITH ARTIFICIAL OXYGEN CARRIERS IN HEMORRHAGED RATS: PROFILES OF HEMOGLOBIN-VESICLE DEGRADATION AND HEMATOPOIESIS FOR 14 DAYS

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ABSTRACT—Polyethylene glycol (PEG)–modified hemoglobin (Hb) vesicles (HbVs) are artificial oxygen carriers encapsulating a concentrated Hb solution in phospholipid vesicles. In our previous studies, HbV showed a sufficient resuscitative effect comparable to that of red blood cells in hemorrhagic shock animal models during several hours' observation. However, the profiles of the recovery, including hematopoiesis and elimination of HbV, remain unknown. This study conducted 14-day observations of Wistar rats after hemorrhagic shock and fluid resuscitation with HbV suspended in recombinant human serum albumin. Shock was induced by 50% blood withdrawal from a femoral artery. The rats showed hypotension, metabolic acidosis, and hyperventilation. After 15 min, they received HbV or shed autologous blood through a femoral vein. Both groups showed rapid recovery of hemodynamic and blood gas parameters. No meaningful difference was found between groups. After decannulation and awakening, the rats were housed in cages. The reduced hematocrit of the HbV group returned to the original level in 7 days. Plasma enzyme levels were slightly higher in both groups at 1 day because of systemic reperfusion injury. Splenomegaly was considerable in the HbV group because of the HbV accumulation and extramedullar hematopoiesis, but it subsided within 14 days. Along with the HbV elimination in the spleen and liver, immunohistochemistry with anti-PEG antibody revealed that PEG-conjugated lipid had disappeared within 14 days. In conclusion, HbV showed a sufficient resuscitative effect comparable to that of red blood cell transfusion. Phagocytized HbV disappeared within 14 days. Elevated hematopoiesis contributed to complete hematocrit recovery within 7 days.

KEYWORDS—Hemorrhagic shock, blood substitutes, transfusion alternative, hemoglobin, liposome, resuscitation, reticuloendothelial system, erythropoiesis

ABBREVIATIONS—β-LP—β-lipoprotein; ALT—alanine aminotransferase; AST—aspartate aminotransferase; BE—base excess; BP—arterial blood pressure; BUN—blood urea nitrogen; CRE—creatinine; GM-CSF—granulocyte macrophage–colony-stimulating factor; HBOCs—hemoglobin-based oxygen carriers; HbV—hemoglobin vesicles; Hct—hematocrit; HSA—human serum albumin; IFN-γ—interferon γ; LDH—lactate dehydrogenase; metHb—methemoglobin; $PaCO_2$—arterial blood carbon dioxide tension; $PaO_2$—arterial blood oxygen tension; PEG—polyethylene glycol; PEG-DSPE—1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG; RBCs—red blood cells; RES—reticuloendothelial system; rHSA—recombinant human serum albumin; SAB—shed autologous blood; UA—uric acid; T-Chol—total cholesterol; WBC—number of white blood cells

## INTRODUCTION

Blood transfusion is an indispensable practice that has contributed substantially to continued improvement of human health and welfare. However, some unresolved issues threaten the achievement of safer transfusions: the possibility of contamination of pathogens such as human immunodeficiency virus and hepatitis virus, or other unknown viruses, even after the costly nucleic acid amplification testing; mismatching of blood types; and numerous immunological difficulties. Guidelines for safer blood transfusion have been revised repeatedly, such as the reduction of a transfusion trigger, the critical hemoglobin (Hb) level, to 6 g $dL^{-1}$ to minimize unnecessary transfusion strictly or to avoid allogeneic transfusion as long as possible to prevent such side effects (1). In this respect, Hb-based oxygen carriers (HBOCs) are superior to allogeneic transfusion because they are free of blood-type antigens, pathogens such as human immunodeficiency virus and hepatitis viruses, and immunological and other blood-bone side effects. The stability of HBOCs for a long-term storage over several years is also advantageous, which far surpasses the limited storage period of packed red blood cells (RBCs), which is limited to a mere 3 weeks in Japan (2).

A phospholipid vesicle or liposome encapsulating concentrated human Hb (Hb-vesicle [HbV]) is one example of an HBOC (3, 4). The cellular structure of the HbV (ca. 250-nm particle diameter) has characteristics resembling those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and

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the endothelial lining, thereby eliminating some side effects of molecular Hbs (5, 6). In previous reports, we clarified that the injected HbVs were finally captured by macrophages in the reticuloendothelial system (RES or mononuclear phagocytic system) and degraded promptly, subsequently disappearing within 2 weeks (7–10). However, those experiments were conducted with healthy rats receiving bolus or repeated topload injections or isovolemic exchange transfusion with less surgical intervention. In exchange hemodilution experiments, hematocrit (Hct) reverted to the original level during 1 week after exchange transfusion with HbV, indicating that the hematopoietic activity was preserved despite the accumulation of HbV in RES, including bone marrow (10). Although we previously confirmed the oxygen transport capacity of HbV as a resuscitative fluid for hemorrhagic shock, the observation period was only several hours (11–14); what would happen during the succeeding days or weeks was not known.

Given this background, the present work is intended to clarify the subsequent profiles of recovery involving the degradation of HbV in RES and hematopoiesis in addition to the oxygen transporting capabilities of HbV and its impact on organ functions compared with resuscitation with shed autologous blood.

## MATERIALS AND METHODS

### Preparation of HbV

For use in this study, HbV was prepared as reported in previous studies (15, 16). The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g $dL^{-1}$) contained 14.7 mM of pyridoxal 5′-phosphate (Sigma Chemical Co., St. Louis, Mo) as an allosteric effector to regulate $P_{50}$ to 25 to 28 torr. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chemical Co. Ltd., Osaka, Japan), along with 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethyleneglycol) (PEG-DSPE, 0.3 mol%; NOF Corp., Tokyo, Japan). The HbVs were suspended in a physiologic salt solution at [Hb] = 10 g $dL^{-1}$ and [lipids] = 6.8 g $dL^{-1}$, and deoxygenated in vials for long-term storage (17). The HbV suspended in saline does not show an oncotic effect, just as RBCs do not. For a large substitution of blood, HbV must be suspended in or coinjected with a plasma substitute to prevent extravasation of the injected fluid and to maintain blood volume. We often use plasma-derived or recombinant human serum albumin (HSA) because the compatibility of HbV and HSA has been confirmed well from rheological points of view (18). Furthermore, in Japan, recombinant HSA (rHSA) will become clinically available in 2008. In this study, the HbV suspension ([Hb] = 10 g $dL^{-1}$; 8.6 mL; occupied volume, 40%) was mixed with an rHSA (25%, 1.4 mL; Nipro Corp., Osaka, Japan) to regulate the concentration of rHSA in the suspending medium to 5 g $dL^{-1}$ and the colloid osmotic pressure to approximately 19 to 20 torr, a physiological colloid osmotic pressure value. Consequently, Hb was 8.6 g $dL^{-1}$; HbV bound oxygen in an aerobic condition. Before use, the suspension was filtered (0.45-μm pore size, Dismic; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to ensure a homogeneous dispersion state.

### Animal model of resuscitation from hemorrhagic shock

The Laboratory Animal Care and Use Committee of the School of Medicine, Keio University, approved the entire experimental protocol. The protocol complies with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council-National Academy of Sciences (Washington, DC: National Academy Press, 1996).

Experiments were carried out using 48 male Wistar rats (274 ± 26 g body weight). The rats were anesthetized using 1.5%-sevoflurane-mixed air inhalation (Maruishi Pharmaceutical Co., Osaka, Japan) with a vaporizer (Model TK-4 Biomachinery; Kimura Medical Instrument Co. Ltd., Tokyo, Japan) throughout the experiment (fraction of inspired oxygen, 21%), whereas spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing; outer diameter, 0.8 mm; inner diameter, 0.5 mm; Natsume Seisakusho Co. Ltd., Tokyo, Japan) filled with a saline solution containing 40 IU $mL^{-1}$ heparin were introduced into the left femoral artery and vein. The arterial catheter was connected to a polygraph system (LEG-1000; Nihon Kohden Corp., Tokyo, Japan).

The systemic blood volume was estimated as 56 mL $kg^{-1}$ body weight (19). Withdrawing 50% of the blood (28 mL $kg^{-1}$, 1 mL $min^{-1}$) from the femoral artery into a heparinized syringe (Fig. 1) induced hemorrhagic shock. The rats were kept hypotensive for 15 min (MAP < 40 mmHg). They were resuscitated by infusion of HbV (n = 24) or shed autologous blood (SAB group; n = 24) at a rate of 1 mL $min^{-1}$. The volume of the infused resuscitative fluid was equal to the shed volume: 50% of the blood volume at baseline. Systemic parameters were observed for 6 h, and then the catheters were removed. The femoral artery and vein were ligated, and the skin was sutured with a stitch. The rats were housed in cages in a room with a barrier against infection at the animal experimental facility of Keio University. Rats were provided *ad libitum* access to food and water in a temperature-controlled environment with a 12-h dark/light cycle.

Five rats were selected randomly from each group at 1, 3, 7, and 14 days for sequential measurements. At each time point, the rats were anesthetized using 1.5%-sevoflurane-mixed air inhalation. After measuring the body weight, approximately 150 μL blood was withdrawn from the tail vein via an indwelling needle (24-gauge; Nipro) for an Hct measurement using glass capillaries and for blood cell counts. A catheter was introduced into the right femoral artery for monitoring systemic hemodynamics and blood gas parameters. The animals were then laparotomized, and approximately 6 mL of blood was withdrawn from the caudal vena cava for plasma biochemical tests. The organs were resected en bloc, weighed, and fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for histochemical examination.

### Measurements

Systemic hemodynamics and blood gases were evaluated before hemorrhage (baseline); after hemorrhage; immediately after resuscitation; and 1, 3, and 6 h after resuscitation with the left femoral artery; and at 1, 3, 7, and 14 days with the right femoral artery. Blood samples were collected in 70-IU $mL^{-1}$ heparinized microtubes (125 μL; Clinitubes; Radiometer A/S, Copenhagen, Denmark) for blood gas analyses and in glass capillaries (Terumo Corp., Tokyo, Japan) for Hct measurements. A pH/blood gas analyzer (either model ABL 555 or ABL 700; Radiometer A/S) was used for analyses of arterial blood oxygen tension ($PaO_2$), carbon dioxide tension ($PaCO_2$), pH, lactate, base excess (BE), and glucose. A recording system (Polygraph System 1000; Nihon Kohden Corp., Tokyo, Japan) was used for continuous monitoring of the arterial blood pressure (BP) and heart rate.

Soon after the measurement of Hct using the glass capillary and centrifugation (12,000 rpm, 5 min), the supernatant (×30 μL) was carefully collected in a plastic tube for measurement of the cytokine levels, HbV concentrations, and methemoglobin (metHb) contents of HbV. The specimens for cytokine measurements were stored in a freezer at −80°C. The concentrations of HbV in the plasma and metHb contents were measured spectroscopically according to a process described in a previous report (17). The collected blood (ca. 6 mL) was centrifuged (5,000*g*, 10 min) to separate the plasma, which was then ultracentrifuged (50,000*g*, 20 min) to sediment the HbV particles from the plasma at 1 and 3 days after resuscitation with HbV to avoid interference by HbV particles in the plasma biochemical assays (20). The obtained transparent serum specimens contained no Hb, indicating that no hemolysis of HbV occurred. The plasma samples of both the HbV and SAB groups and those from 10 healthy rats as the control group were stored at −80°C until biochemical tests were conducted at BML, Inc. (Kawagoe, Japan).

### Measurements of cytokine

Plasma cytokine levels were measured using a Bioplex Protein Array system (Bio-Rad Laboratories Inc., Hercules, Calif) according to the manufacturer's instructions. This is a multiplexed, particle-based, flow cytometric assay that uses anticytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes (21). The assay enables quantification of several mediators in a sample volume as small as 15 μL. Our assay was customized to detect and quantify rat IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNF-α, interferon (IFN) γ, and granulocyte macrophage–colony-stimulating factor (GM-CSF). For each cytokine, eight standards were between 2 and 32,000 pg $mL^{-1}$.

### Histopathological examination

The organs were fixed in a 10% formalin neutral buffer solution (Wako). The paraffin sections were stained using hematoxylin/eosin and by Berlin blue method. Immunohistochemical analyses of spleen and liver tissues were performed to detect the released PEG chains that are conjugated on the HbV surface.

Subsequently, 4-μm-thick paraffin sections were treated with 0.3% $H_2O_2$ in methanol for 20 min. After blocking nonspecific binding with an antibody diluent (S2022; DakoCytomation, Glostrup, Denmark), they were incubated overnight at 4°C with rabbit monoclonal antibody [PEG-B-47] against methoxy PEG (1/200 dilution with a diluent, ab51257; Abcam PLC, Cambridge, England). They were then incubated for 45 min at room temperature with antibodies against mouse and rabbit immunoglobulins conjugated to the amino acid polymer (no dilution, Histofine Simple Stain MAX-PO(MULTI), Code: 414191; Nichirei Corp., Tokyo, Japan). Negative control was performed without the primary antibody against PEG.

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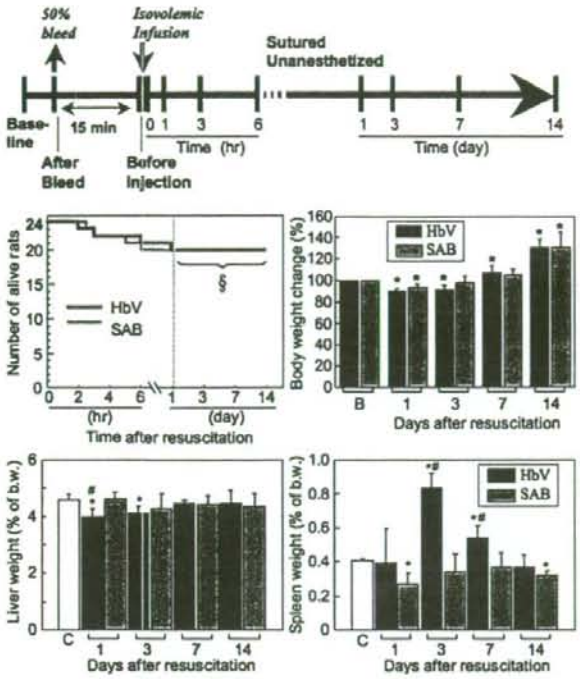


FIG. 1. **Scheme illustrating the experimental protocol (top). Number of rats that after resuscitation from hemorrhagic shock with HbV or SAB (middle left).** The 20 rats survived hemorrhagic shock and resuscitation for 6 h in an anesthetized condition. The catheters in the femoral artery and vein were removed, and the rats were unanesthetized and housed in cages. §During this period, all the rats survived until the planned killing (survival rate, 100%) after nonanesthesia for up to 14 days. Among 20 rats, 5 rats each were killed for examination at 1, 3, 7, and 14 days. **Body weight changes (%) of Wistar rats after resuscitation from hemorrhagic shock with HbV or SAB (middle right).** Mean ± SD. No significant difference was found between the groups ($n = 5$). *$P < 0.05$ vs. baseline. B, Baseline. **Weight ratios of liver and spleen to body weight at 1, 3, 7, and 14 days after resuscitation with HbV or SAB (bottom).** Mean ± SD. *$P < 0.05$ vs. baseline; #$P < 0.05$ vs. SAB ($n = 5$). C, Control group. Five healthy rats were used for the control group.

Color was developed using 3,3'-diaminobenzidine (16.7%; Sigma) in 0.05 M Tris-HCl, pH 7.4, containing 0.04% $H_2O_2$. Nuclei were stained with hematoxylin.

Our separate studies clarified that the PEG molecule itself (MW, 5,000) is very soluble in both water and organic solvents, and it easily disappears from the tissue sections during the staining procedures. On the other hand, PEG-DSPE remains in the sections probably because released PEG-DSPE would be anchored to any cell membranes or proteins.

### In vivo *data analysis*

The *in vivo* data are given as the mean ± SD for the indicated number of animals. Data were analyzed using StatView (ver. 5.0; Abacus Concepts, Inc., Berkeley, Calif). For systemic parameters, time-related differences compared with the baseline within each group were assessed using a paired *t* test. Differences among the groups at the same time point were assessed using ANOVA, followed by Fisher protected least significant difference. Unpaired *t* tests were used for comparison of plasma enzyme levels between the groups. Differences were inferred as significant when $P < 0.05$.

## RESULTS

### *Survival rate and body weight increase*

Among the 24 rats that received HbV as a resuscitative fluid, 20 rats survived the 6-h anesthetized condition. The catheters in the femoral artery and vein were removed. The rats were unanesthetized and housed in cages. All rats, after awakening from anesthesia, survived up to 14 days later until their planned death (Fig. 1). This is a very similar tendency to that of the rats that received SAB. The body weights of both the HbV and SAB groups (baseline, 274 ± 21 and 287 ± 41 g, respectively) had decreased by 10% at 1 day after resuscitation. However, the rats then tended to gain weight, as measured at 3 days; finally, their weight increased to approximately 130% of the baseline levels. No significant difference was found between the two groups.

### *Organ weights*

The spleen weight ratio to the body weight had increased significantly in the HbV group by 3 days (Fig. 1). Splenomegaly was attributable mainly to the accumulation of HbV; the maximum ratio was observed at 3 days. However, it had reverted to the baseline level by 14 days. The spleen of the SAB group showed no such changes; however, some shrinkage was observed at 1 day probably because of autotransfusion. The liver-weight-to-body-weight ratios of both groups showed no such remarkable changes. However, the HbV group showed significant reduction by 1 and 3 days after resuscitation.

### *Systemic parameters*

Arterial blood pressure before hemorrhage was 84 ± 8 mmHg on the average; it decreased significantly to 25 ± 6 mmHg immediately after hemorrhage. It increased slightly to 39 ± 12 mmHg after 15 min (immediately before resuscitation; Fig. 2). After resuscitation, both the HbV and SAB groups showed immediate recovery beyond the baseline level. The SAB group showed significantly higher BP than HbV. At 1 h, BP was reduced to approximately 75 mmHg and remained stable for 6 h. Overshooting probably occurred because of hypervolemia at the initial phase caused by the combination of autotransfusion, isovolemic injection, and monitoring of peripheral, not central, blood pressure. BP was stable for the succeeding 14 days in both groups. The heart rate (baseline, 414 ± 41 beats per minute) tended to show a slight reduction during shock and at 0, 3, and 6 h after resuscitation. However, both groups showed stable values for the 6 h of anesthesia and for the succeeding 14 days.

The values of $PaO_2$ (baseline, 81 ± 8 torr) increased to 109 ± 8 torr on the average. In addition, $PaCO_2$ (baseline, 40 ± 5 torr) decreased to 28 ± 5 torr after hemorrhage because of compensatory hyperventilation. The lactate level increased from 1.2 ± 0.4 to 5.3 ± 1.5 mM immediately after hemorrhage and to 8.0 ± 2.2 mM after 15 min. Metabolic acidosis (pH 7.26 ± 0.09) became evident 15 min after hemorrhage (baseline, 7.44 ± 0.03). The BE (baseline, 2.7 ± 1.4 mM) had decreased significantly to −11.7 ± 4.2 mM by 15 min after hemorrhage. The blood glucose level also increased from 9.8 ± 0.7 mM as the baseline level to 23.0 ± 2.4 mM 15 min after hemorrhage. Immediately after resuscitation, $PaO_2$ and $PaCO_2$ reverted to the baseline level. However, pH, lactate, and glucose levels showed a complete but delayed recovery 1 h after resuscitation. Furthermore, BE showed incomplete recovery 1 h after resuscitation; complete recovery was achieved at 1 day. These parameters were stable

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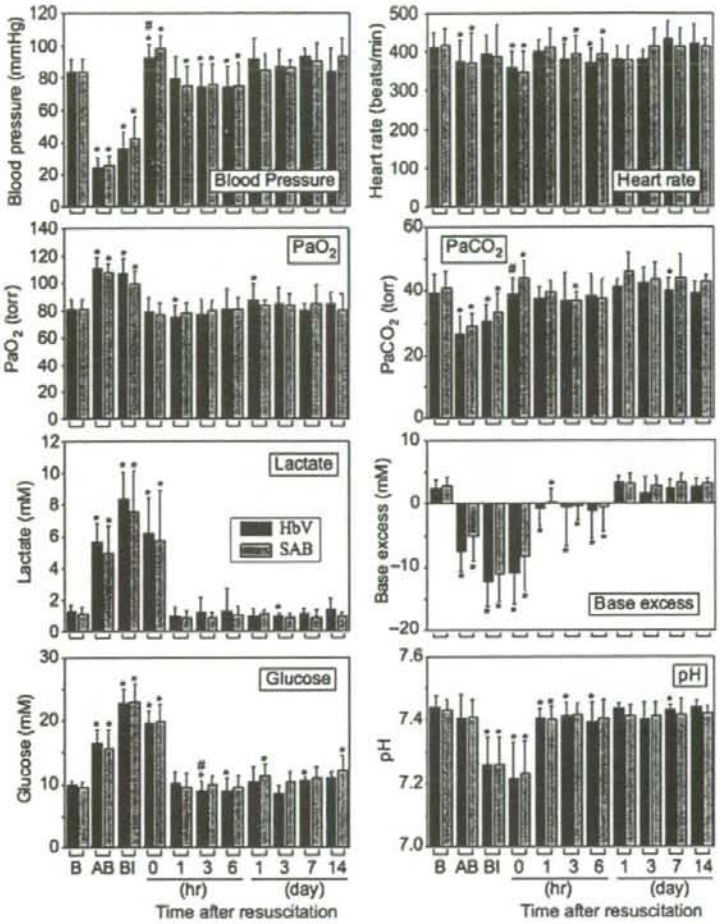


FIG. 2. **Hemodynamic and blood gas parameters during and after hemorrhagic shock and resuscitation with HbV or SAB for 6 h in anesthetic condition and the succeeding 14 days.** Mean ± SD. $^{*}P < 0.05$ vs. baseline. $^{\#}P < 0.05$ vs. SAB (B, 6 h; n = 24; 1–14 days; n = 5). AB indicates after bleeding; B, baseline; BI, before injection.

after the anesthetic condition and 14 days of observation. No meaningful difference was found between the groups.

Hematocrit had decreased from the baseline, 40% ± 2%, to 30% ± 3% at 15 min after blood withdrawal because of the dilution of the remaining blood by autotransfusion (Fig. 3). After resuscitation with SAB, Hct increased to 36% ± 2% immediately after resuscitation; it further increased to 41% ± 3% 1 h after resuscitation. It sustained a higher level for 14 days. Immediately after resuscitation with HbV, Hct was further reduced to 20% ± 7% as a result of the dilution of blood; it remained stable for 6 h. Subsequently, it tended to increase and reverted to the original level 7 days after resuscitation and to a slightly higher level at 14 days. The number of platelets decreased markedly after resuscitation with HbV, as did Hct, probably as a result of the dilution of blood, but it returned to the baseline level 3 days after resuscitation, which was earlier than the recovery of Hct. The number of white blood cells (WBCs) showed a slight reduction after resuscitation with HbV, but it increased at 6 h. Large deviations were apparent in WBC; no statistically significant difference was found. The Hb concentration derived from HbV immediately after resuscitation was 3.6 ± 0.8 g $dL^{-1}$. It did not change until 6 h (3.6 ± 0.5 g $dL^{-1}$). However, it had decreased to 2.8 ± 0.6 g $dL^{-1}$ at 1 day, 1.6 g $dL^{-1}$ at 3 days, and to an undetectable level at 7 days. The metHb content of the HbV fraction elevated to 37% ± 3% at 6 h and 83% ± 4% at 1 day.

### *Plasma biochemical parameters*

One day after resuscitation, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) tended to increase in both groups in comparison to the baseline levels (Fig. 4). However, they tended to return to the baseline level 3 days after resuscitation. The total protein concentration showed minimal changes. Amylase levels decreased significantly for both groups in comparison to the baseline levels. However, lipase levels displayed no such changes. Creatinine (CRE), uric acid (UA), and blood urea nitrogen (BUN) exhibited marked changes that were not statistically significant. Both groups showed reduction of

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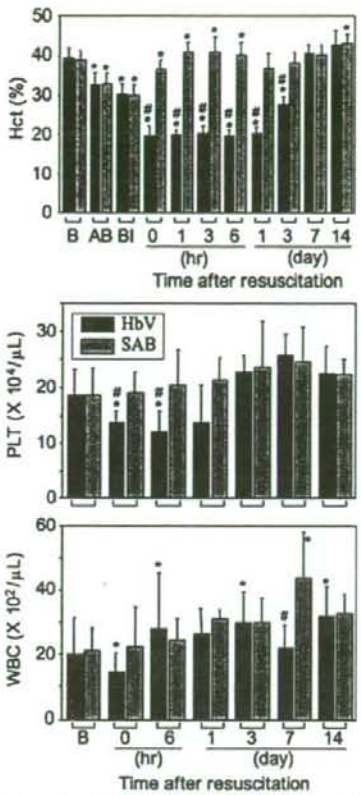


FIG. 3. **Hematocrit and the numbers of platelets and WBCs during and after hemorrhagic shock and resuscitation with HbV or SAB for 6 h in anesthetic condition and the succeeding 14 days.** Mean ± SD. $^*P < 0.05$ vs. baseline. $^\#P < 0.05$ vs. SAB (B, 6 h, n = 24; 1–14 days; n = 5). AB indicates after bleeding; B, baseline; BI, before injection.

plasma $Fe^{3+}$ level for 3 days and reverted to the baseline level in 14 days. The return was faster for the SAB group than for the HbV group. The total bilirubin level remained at low levels throughout the experiment (<0.1 mg $dL^{-1}$ detection limit). The plasma total cholesterol (T-Chol) and β-lipoprotein (β-LP) levels had increased significantly for the HbV group at 1 and 3 days after resuscitation, but the phospholipid levels were decreased in both groups. The increased T-Chol and β-LP returned to the baseline levels at 7 days. The SAB group showed no such changes. Erythropoietin activity in the HbV group was significantly higher than that of the SAB group at 1 and 3 days.

### *Plasma cytokine levels*

Neither group showed an increase in IL-1α, IL-2, IL-4, IL-6, IFN-γ, or GM-CSF in comparison to the baseline levels (Fig. 5). Slight increases were apparent for IL-1β for the SAB group (<20 pg $mL^{-1}$), IL-10 for both groups (<50 pg $mL^{-1}$), and TNF-α for the HbV group (<50 pg $mL^{-1}$) after resuscitation. However, they were much lower than those in a septic shock model induced using an LPS injection (IL-1β, 8,000 pg $mL^{-1}$; TNF-α, 4,000 pg $mL^{-1}$; IL-10, 1,200 pg $mL^{-1}$), which were confirmed in our unpublished data and in a previous report (22).

### *Histological examination*

Sections of the spleen and liver of the HbV group showed accumulation of HbV particles in the spleen macrophage in the red pulp zone and liver Kupffer cells 1 day after resuscitation, as portrayed in Figure 6. At 3 days, many nests of erythroblasts were observed in the red pulp zone in the spleen, indicating aggravated extramedullar erythropoiesis. At 7 days, Kupffer cells phagocytizing HbV were not detectable. However, many spleen macrophages phagocyting HbV were observed. At 14 days, HbV disappeared in the spleen. The Berlin-blue method indicated the presence of hemosiderin in macrophages of the spleen in the HbV group at 14 days but not at 1, 3, and 7 days (data not shown). Hemosiderin deposition was undetected in the liver. No other abnormal morphological changes were revealed by our examinations.

Figure 7 portrays immunohistochemical staining of spleen and liver tissues with anti-PEG antibody. For the liver, PEG was detectable 1 day after resuscitation. The strongest deposition was observed at 3 days, but it was not detectable at 14 days. The spleen, on the other hand, showed no staining at 1 day; the strongest deposition was observed at 7 days. It was speculated that PEG of PEG-DSPE became detectable during degradation of HbV. At 14 days, no staining was detected in the spleen.

## DISCUSSION

Our primary finding in this study is that HbV showed a similar resuscitative ability to that of SAB, as evidenced by the similar survival rate and levels of systemic parameters after resuscitation for 14 days. The reduced Hct reverted to the original level in 7 days, and the phagocytized HbV seemed to disappear during the observation period.

Experimental hemorrhage induced significant hypotension, hyperventilation, lactacidemia, glycemia, and acidosis. Glycemia is induced by accelerated glycogenolysis in the liver because of catecholamine secretion elicited via baroreceptors in hemorrhagic shock (23, 24). In both HbV and SAB groups, these parameters reverted completely to their respective baseline levels within 1 h after resuscitation, although both groups included four death cases among the 24 rats. Hemorrhagic shock and resuscitation induce ischemia, hypoxia, and reperfusion injury, all of which influence organ functions. Many precedent articles have described the elevation of plasma enzyme levels, AST, ALT, and LDH after resuscitation with HBOCs or transfusion (13, 25–29). Therefore, such elevation would unquestionably occur after resuscitation with HbV or SAB. The plasma enzymatic activities of amylase and lipase, which reflect the function of pancreas, and the levels of CRE, UA, and BUN, which reflect the renal function, showed some significant changes. Nevertheless, they did not seem to constitute meaningful changes in this experiment. The results of the elevated AST, ALT, and LDH raise the issue of the resuscitation treatment. Resuscitation with an oxygen-carrying fluid induces oxidative damage at an early phase; blood volume recovery is primarily important. Slight increases in IL-10 and TNF-α were confirmed, although the levels were very small in comparison

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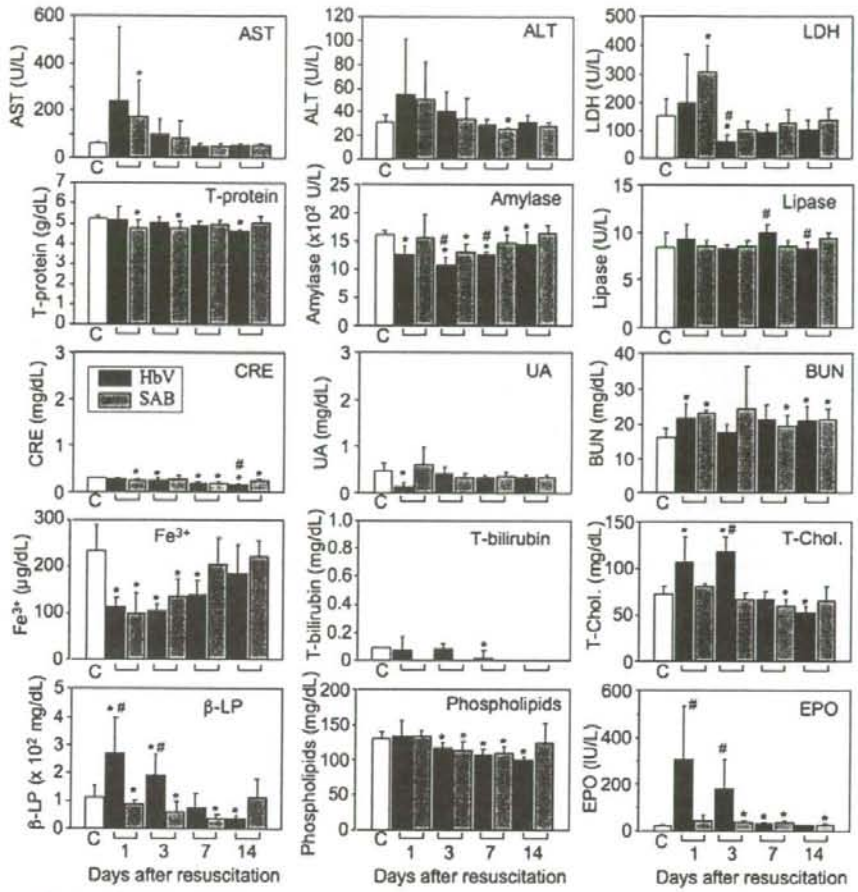


FIG. 4. **Plasma enzyme levels at 1, 3, 7, and 14 days after resuscitation with HbV or SAB.** Mean ± SD. *$P < 0.05$ vs. control group (n = 5). #$P < 0.05$ vs. SAB group. C, Control group. Ten healthy rats were used for the control group. EPO indicates erythropoietin; T-bilirubin, total bilirubin; T-protein, total protein.

to the significant elevation that can result from endotoxemia (data not shown), which is probably a response to light inflammatory damage induced by reperfusion.

The liver is an important organ for degradation of HbV in RES. We anticipated that such an oxidative situation might reduce the capacity of degradation of HbV in RES. Pathological examination of the liver showed evidence of Kupffer cells phagocytizing HbV; it disappeared within 7 days in the liver. In the spleen, substantial accumulation of HbV was confirmed in macrophages in the red pulp zone in the same manner as that in previous studies of bolus injection, daily repeated injections, and exchange transfusion (7, 9, 13). It is reported that PEG-conjugated Hb, one HBOC developed in the United States, also accumulates in the spleen; foamy or vacuolated macrophages were observed (29, 30). Spleen macrophages serve an important role of eliminating a series of HBOCs from blood circulation (31). It must be clarified that no components are deposited in the tissue for a long time. In our previous studies of toploud infusion of HbV (20 mL $kg^{-1}$, [Hb] = 10 g $dL^{-1}$; 2 g Hb $kg^{-1}$) and 40% blood exchange (22.4 mL $kg^{-1}$, i.v., [Hb] = 8.6 g $dL^{-1}$; 1.9 g Hb $kg^{-1}$) in healthy rats, the HbV disappeared within 7 days (9, 10). However, in the present study, HbV remained using the time of measurement at 7 days but had disappeared at 14 days. One reason for the slow degradation might be that the dosage is calculated as 28 mL $kg^{-1}$ ([Hb] = 8.6 g $dL^{-1}$; 2.4 g Hb $kg^{-1}$), which is slightly greater than that used in the precedent reports. Another reason is that phagocytosis would be compromised to some degree in this shock model, although HbV disappeared at 14 days. The possibility exists that the distribution of HbV is different in this shock model from the distribution shown for toploud injection to normal healthy rats. Our collaborators are studying details of the biodistribution.

The gradual increase in the plasma total cholesterol levels by 3 days after resuscitation suggests that the cholesterols are liberated from the RES after the HbVs are captured by the RES and destroyed in the phagosomes of the macrophages (7, 8). In our previous studies of toploud HbV infusions, significant increases in the high-density lipoprotein–cholesterol, β-LP, and phospholipids were observed as surplus amounts (8, 9). In contrast, we observed no such increase resembling those that were apparent after the 40% blood exchange and the present resuscitation studies—only

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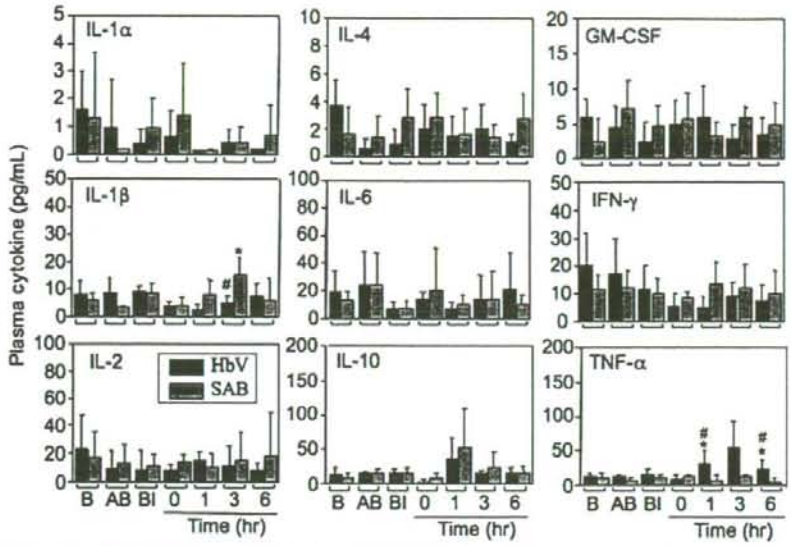


FIG. 5. **Plasma cytokine levels during hemorrhagic shock and a 6-h resuscitation period.** Neither group showed an increase in IL-1α, IL-2, IL-4, IL-6, IFN-γ, or GM-CSF in comparison to the baseline levels. Slight increases were apparent for IL-1β for the SAB group at 3 h, IL-10 for both groups at 1 h, and TNF-α for the HbV group at 3 h after resuscitation. However, they are much lower than those obtained using a septic shock model induced with an LPS injection (IL-1β, 8,000 pg $mL^{-1}$; TNF-α, 4,000 pg $mL^{-1}$; IL-10, 1,200 pg $mL^{-1}$), as described in unpublished data and an earlier report (22). Mean ± SD. $^{*}P < 0.05$ vs. baseline. $^{\#}P < 0.05$ vs. SAB.

decreases (10). A large demand for nutrients is expected to pertain for hematopoiesis; in addition, the lipid components from HbV can be used efficiently for proliferation. The plasma bilirubin levels and $Fe^{3+}$ showed no abnormal increases, although hemosiderin deposition was detected in the spleen, but not in the liver, of the HbV group at 14 days. Because of the shorter half-lives of the stored RBCs, hemosiderosis is often observed in patients who have received repeated blood transfusions. These results indicate that no obstruction or stasis of the bile is expected to occur in the biliary tree, and that the metabolism of heme from HbV and the iron storage is within physiological capacity (10).

The HbV surface is modified with PEG chains by using the PEG-conjugated phospholipid PEG-DSPE. They stabilize the dispersion state during preservation and in blood circulation. It is expected that PEG-DSPE is hydrolyzed at the crosslink between the PEG chain and DSPE; the released PEG would be excreted through the kidney (32). The weight percentage (wt%) of PEG in the total chemical components of HbV (Hb and lipids) is estimated as only 1 wt%, which is considerably less than that of PEG-Hb (32 wt%); the latter comprises one Hb and six PEG strands (33). However, our previous studies of HbV did not confirm whether PEG disappears from RES. We attempted immunohistochemical analyses using rabbit anti–methoxy-PEG antibody for the first time to collect some information. Kupffer cells showed staining with the anti–methoxy-PEG antibody 1 day after resuscitation, but not in the spleen macrophages. The strongest staining was observed 7 days after resuscitation in the spleen. These results indicate that PEG chains conjugated on the surface of HbV are not recognized using the antibody in a similar manner as the excluded volume of PEG chains on the surface of HbV prevents the access of macromolecules, plasma proteins, and enzymes. During the degradation of HbV, the PEG chains of PEG-DSPE became recognizable with the antibody. At 14 days, PEG was undetectable presumably because the conjugate between the PEG and DSPE was hydrolyzed and PEG would be excreted through the kidney. A detailed biodistribution study is necessary to show complete excretion.

Transient but substantial accumulation of HbV in RES raises concerns of the impact on the ability of the RES to respond to infectious challenge. We previously measured the phagocytic activity by carbon clearance measurement after injection of HbV to normal rats (7). The phagocytic activity decreased transiently 1 day after HbV infusion (20 mL $kg^{-1}$) by approximately 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the quiescent level at 7 days. It then returned to the normal value at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. It does not seem to cause any irreversible damage to the phagocytic organs. These results are demonstrated only in healthy rats, although rats in hemorrhagic shock or septic shock and those with repeated infusions might react differently in these pathological situations. These results must be clarified through ongoing safety evaluations.

The reduced Hct in the HbV group returned to its original level at 7 days because of the aggravated hematopoiesis, which was evident from the large amount of nests of erythroblasts in the red pulp zone in the spleen. At the same time, the phagocytized HbV was degraded and disappeared. Because of the rapid metHb formation and short circulation half-life, the HbV group became anemic, and erythropoietin excretion was enhanced, which might have facilitated hematopoiesis in the spleen. In rats, extramedullary hematopoiesis induced by hypoxia is localized predominantly in the spleen

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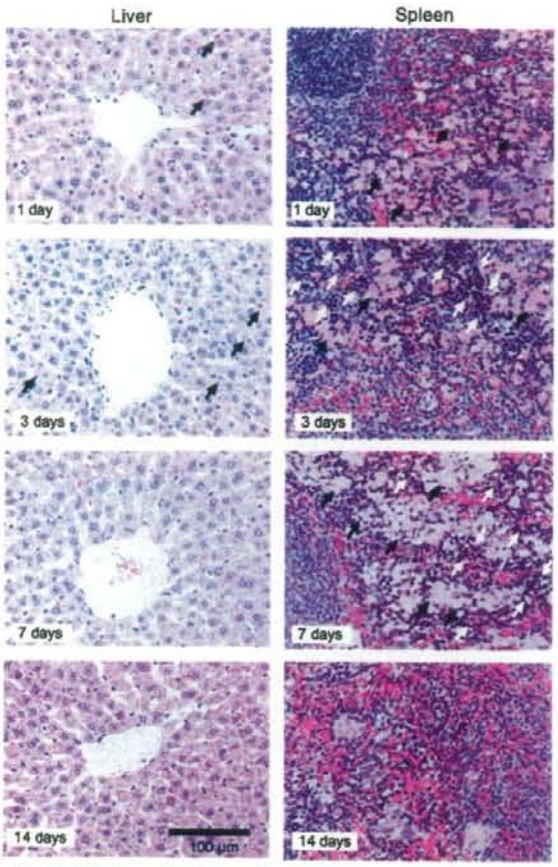


FIG. 6. **Histology of rat liver and spleen of HbV group at 1, 3, 7, and 14 days after resuscitation.** Hematoxylin and eosin staining. Black arrows indicate the presence of spleen macrophages and liver Kupffer cells phagocytizing HbV particles. Hemoglobin vesicles disappeared completely at 14 days. The white arrows in spleens indicate nests of erythroblasts in the red pulp zone, reflecting enhanced extramedullar erythropoiesis.

(34, 35). Intravenous administration of HbV can restore hemorrhagically shocked animals in a critical condition (emergency care). However, the functional half-life of HbV is much shorter than that of RBC. For that reason, additional infusion of HbV or RBC would be necessary to minimize the anemic period. Regarding the safety of repeated infusion of HbV, we previously tested massive doses of HbV to healthy rats using repeated infusions (10 mL $kg^{-1}$ $d^{-1}$ × 14 d). Although the experimental model was not relevant to the present study and clinical situation, all rats survived, and no severe toxicity was confirmed except splenohepatomegaly, hemosiderin deposition, and lipidemia (8). However, we must confirm the safety of repeated infusions of HbV in a hemorrhagic shock model.

In fact, HbV contains no enzymes such as catalase, superoxide dismutase, or metHb-reducing enzymes that are originally present in RBCs. During our rigorous purification process of the Hb solution from outdated RBCs (virus inactivation by heating and virus removal by nanofiltration) (16), we eliminated all unstable enzymes, aiming at utmost safety from infection. The advantages of HbV are that it is pathogen-free and blood-type-antigen-free; moreover, it can withstand long-term storage of a few years, none of which can be achieved by the conventional blood transfusion systems. As a result, the metHb formation is fast, and functional half-life is short. This profile is common to all HBOCs, and we must select suitable clinical uses. In this study, we examined the possible use of HbV as a resuscitative fluid for hemorrhagic shock temporarily, as during an emergency situation, or for bridging until RBCs are available. Other possible uses might be (1) as a fluid for preoperative hemodilution or as a perioperative oxygen supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion; (2) as a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery; and (3) as an alternative for use for other potential indications, for example, so-called oxygen therapeutics to oxygenate ischemic tissues (2).

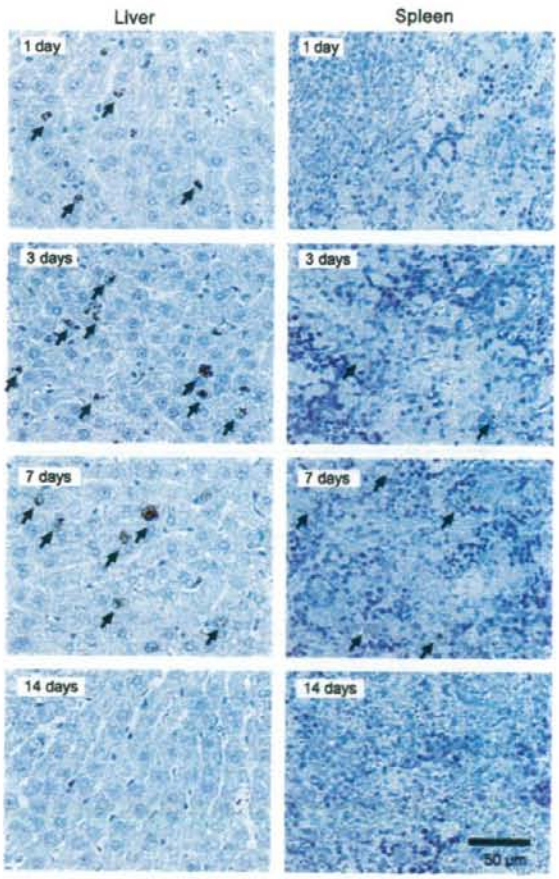


FIG. 7. **Immunohistochemical staining with anti-methoxy-PEG antibody.** Black arrows indicate stains in the liver Kupffer cells and spleen macrophages. Staining was detectable at 1 day in the liver, but not in the spleen, although the amount of HbV was much larger in the spleen. In the spleen, the strongest staining was observed at 7 days after resuscitation. These results show that the anti-methoxy-PEG antibody had difficulty in recognizing PEG on the surface of HbV; also, degradation of HbV in the spleen macrophage is much slower than that in the Kupffer cells.

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In summary, resuscitation with HbV suspended in rHSA showed rapid recovery of hemodynamic and blood gas parameters. They were stable for 14 days. The profiles were identical to those of SAB. The HbV group gained body weight, and their reduced Hct returned to its original level by 7 days because of elevated hematopoiesis. Significant splenomegaly was observed in the HbV group at 3 days because of HbV accumulation. However, it subsided within 14 days. Histopathologically, a significant amount of HbV accumulated in the spleen macrophages, with complete disappearance within 14 days, and HbV induced no overt toxicity. Based on results of this study, we began similar studies using beagle dogs and observed detailed hemodynamic parameters in addition to long-term survival and safety outcomes. Those studies' results will be reported elsewhere.

## ACKNOWLEDGMENTS

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# Enhanced radiation response of a solid tumor with the artificial oxygen carrier 'albumin-heme'

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**Tumor-cell hypoxia is one of the main factors inducing radioresistance. Enhanced tumor oxygenation has previously been achieved in an animal model using the synthetic heme-based oxygen carrier 'albumin-heme' (recombinant human serum albumin-Fe cyclohexanoil heme; rHSA-FeP). The present study was done to determine whether rHSA-FeP enhances the radiation response in an experimental tumor model. Male Donryu rats and LY80, a variant of the syngenic liver ascites tumor, were used. A total of $1 \times 10^6$ cells were injected into the subfascial tissue of the right thigh. The rats were divided randomly into five groups: sham (tumor implantation and sham operation); rHSA-FeP; irradiation; rHSA + irradiation; and rHSA-FeP + irradiation. Six days after, under general anesthesia, intra-arterial administration of 10 mL/kg of either 5% rHSA solution or oxygenated rHSA-FeP solution at 2.5 mL/min was done and a dose of 20 Gy was given. There were significant differences in tumor growth between the sham and irradiation groups, and between the sham and rHSA-FeP + irradiation groups. Tumor growth delay was observed and differences were significant between the sham and irradiation groups, and between the irradiation and rHSA-FeP + irradiation groups. In the present study, rHSA-FeP itself had a slight effect on tumor growth without irradiation. Enhancing the effect of rHSA-FeP on the radiation response is responsible in part for the oxygen-carrying property of rHSA-FeP. In conclusion, rHSA-FeP is a candidate radiation-enhancing drug. Arterial infusion of rHSA-FeP may serve as a local oxygenation method that enhances the radiation effect. (*Cancer Sci* 2008; 99: 1274–1278)**

Resistance to radiation therapy is observed in many types of tumors and can be due to several causes.[1,2] Tumor cell hypoxia and tumor cell repopulation are the main factors causing radioresistance. Oxygen mediates the majority of biological effects of sparsely ionizing radiation, and the response of cells to radiation depends strongly on the availability of oxygen.[3] Various methods to deliver oxygen to cancer tissue have been studied, including inhalation of high oxygen-content gas,[4] the use of an artificial oxygen carrier,[5–7] and the use of agents that manipulate tumor blood flow.[8] Some of these methods have been studied in the clinical setting.[9,10]

Tumor circulation is characterized by tortuous capillaries, compressed vessels, a scant capillary network, arterio-venous junctions, and plasma channels. It is difficult to deliver adequate oxygen to hypoxic cancer tissue as the tortuous circulation causes limited flow of red blood cells. We developed a synthetic heme-based molecular oxygen carrier, albumin-heme (rHSA-FeP), which is recombinant human serum albumin (rHSA) incorporating a Fe (II)tetra(phenyl)porphyrin derivative (FeP) (Fig. 1). We found that this molecule has the potential to transport oxygen *in vivo*.[11] As for the tumor tissue oxygen status, a hypoxic environment is usual in experimental tumor models as well as in clinical cases. Thus, we analyzed the tissue oxygen tension in the transplanted tumor in a previous report.[12] LY80 was evaluated using the Pd coproporphyrin phosphorescence decay method and we found that intra-arterial injection of rHSA-FeP can increase tissue oxygen tension 2.4 times higher than rHSA solution.

In the present study, we studied whether rHSA-FeP could enhance the response to radiation therapy in an experimental tumor model.

## Materials and Methods

**Animal and tumor.** Five-week-old male Donryu rats (Crj-Donryu; Nippon Charles-River, Yokohama, Japan), with an average weight of 120–150 g, were used in the present study. The rats (two to three per cage) were maintained on a bed of pulp paper in a ventilated, temperature-controlled (23 ± 1°C), specific pathogen-free environment with a 12:12 h L:D cycle and access to food and water ad libitum.

The LY80 tumor cell, a variant of the Yoshida sarcoma, was used for this study. LY80 was established in 1966 by Hiroshi Satoh and has been maintained by successive intraperitoneal transplantation. This tumor was kindly donated by Dr Katsuyoshi Hori of Tohoku University. The characteristics of this tumor have been described by Tanda *et al.*[13] Briefly, this tumor was a subline of ascites hepatoma. Once transplanted intraperitoneally, tumor cells grew in ascites and caused peritoneal dissemination as well as systemic spread. Solid tumors may be obtained by subcutaneous transplantation of tumor cells.

All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at our university and were carried out in accordance with the Guidelines for Animal Experiments issued by Keio University School of Medicine, Experimental Animal Center, and law no. 105 and notification no. 6 issued by the Japanese Government. The ethical guidelines that were followed meet the guidelines for animal handling issued by the United Kingdom Co-ordinating Committee on Cancer Research, 1998.

**Tumor cell implantation.** LY80 cells growing in the peritoneal effusion of a donor rat were collected, suspended in phosphate-buffered saline, and adjusted to a concentration of $10^7$ cells/mL. Recipient rats were anesthetized with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). A small skin incision was made on the lateral side of the inguinal ligament of the right thigh. The tumor cell suspension was drawn into a 1-mL graduated syringe (Nipro, Osaka, Japan), and a 30-gauge syringe needle (Becton Dickinson, Franklin Lakes, NJ, USA) was used to inject 100 μL of the cell suspension directly under the subfascial tissue of the biceps muscle, avoiding the femoral artery and vein. The incision wound was then closed with one layer of thin silk thread, and the animals were allowed to recover.

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