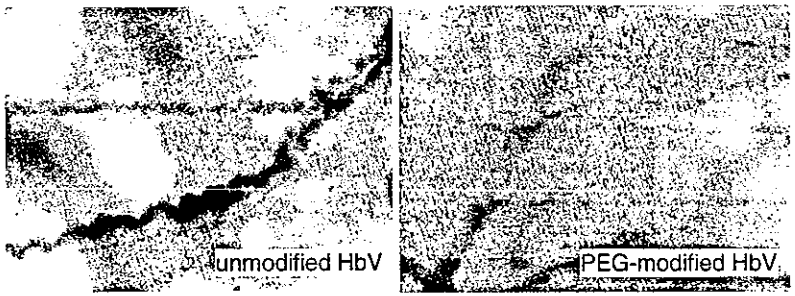

FIG. 8. Micrographs of microvasculature after 80% exchange transfusion with unmodified HbV or PEG-modified HbV suspended in 5% HSA. A high contrast was obtained by illumination with a wavelength range of around 420 nm, being absorbed at the Soret band of Hb molecules in HbV and RBC. Left, the aggregated vesicles block and chains in collecting venules were observed. Right, the microvasculature of postcapillaries is blackened owing to the homogeneous dispersion of PEG-modified HbV particles in the plasma phase. This is effective for better blood flow in the microcirculation

achieved with HSA alone. Subcutaneous microvascular studies showed that PEG-modified HbV/HSA significantly improved microhemodynamic conditions (flow rate, functional capillary density, vessel diameter, and oxygen tension) relative to unmodified HbV/HSA. PEG-modified HbV was homogeneously dispersed in the plasma phase while the unmodified HbV showed aggregation in venules and capillaries. PEG reduced vesicular aggregation and viscosity, improving microvascular perfusion relative to the unmodified type. However, the microvascular perfusion with PEG-modified HbV/HSA was lower than the blood perfused one.

Improved Oxygenation in Ischemic Hamster Flap Tissue by Hemodilution with HbV [107]

Objective. The aim of this study was to test the influence of oxygen affinity of HbVs and level of blood exchange on the oxygenation in collateralized, ischemic, and hypoxic hamster flap tissue during normovolemic hemodilution.

Methods. Microhemodynamics were investigated with intravital microscopy. Tissue oxygen tension was measured with Clark-type microprobes. HbVs with a P_{50} of 15 Torr (HbV_{15}) and 30 Torr (HbV_{30}) were suspended in 6% Dextran 70 (Dx70). The Hb concentration of the solutions was 7.5 g/dl. A stepwise replacement of 15%, 30%, and 50% of total blood volume was performed, which resulted in a gradual decrease in total Hb concentration.

Measurements and Main Results. In the ischemic tissue, hemodilution led to an increase in microvascular blood flow to maximally 141%–166% of baseline in all groups (median; $P < 0.01$ vs. baseline, not significant between groups). Tissue oxygen tension was transiently raised to 121 ± 17% after the 30% blood exchange with Dx70 ($P < 0.05$), whereas it was increased after each step of hemodilution with HbV15-Dx70 and HbV30-Dx70, reaching 217 ± 67% ($P < 0.01$) and 164 ± 33% ($P < 0.01$ vs. baseline and other groups), respectively, after the 50% blood exchange. From these results it can be concluded that despite a decrease in total Hb concentration, the oxygenation in the ischemic, hypoxic tissue could be improved with increasing blood exchange with HbV solutions. Furthermore, better oxygenation was obtained with the left-shifted HbVs.

Safety of HbV (In Vitro and In Vivo Tests)

Rheological Property and Oxygen Releasing Behavior

The rheological property of an artificial oxygen carrier is important because the infusion amount should be significantly large and that may affect the blood viscosity and hemodynamics. It has been suggested that the higher viscosity and the resulting higher perfusion pressure would be beneficial to increase the shear stress on the vascular wall for vasorelaxation and to homogeneously transmit the pressure to microvascular networks and thus to supply blood to whole capillaries [108]. PEG-modified HbV suspended in 5% HSA solution was mixed with human blood and the viscosity was measured. The viscosity was similar to that of blood, and the mixtures at various mixing ratios showed a viscosity of 3–4 cP. RBC is the main component to determine blood viscosity and the results indicate no significant interaction between HbV and RBC [39]. To observe the flow pattern of the mixture of HbV and RBC, they were mixed in various volume ratios at [Hb] = 10 g/dl in isotonic saline containing 5% HSA, and the suspension was perfused at the centerline flow velocity of 1 mm/s through an O_2 permeable fluorinated ethylenepropylene copolymer tube (inner diameter, 28 μm) exposed to a deoxygenated environment [109]. The mixtures of acellular Hb solution and RBC were also tested. Since HbV was homogeneously dispersed in the HSA solution, increasing the volume of the HbV suspension resulted in a thicker marginal RBC-free layer (Fig. 9).

In the same experimental model, measurement of the O_2 release from the narrow tube was performed using a scanning-grating spectrophotometer with a photon count detector, and the rate of O_2 release was determined based on the visible absorption spectrum in the Q band of Hb [109]. Irrespective of the mixing ratio, the rate of O_2 release from the HbV-RBC mixtures was

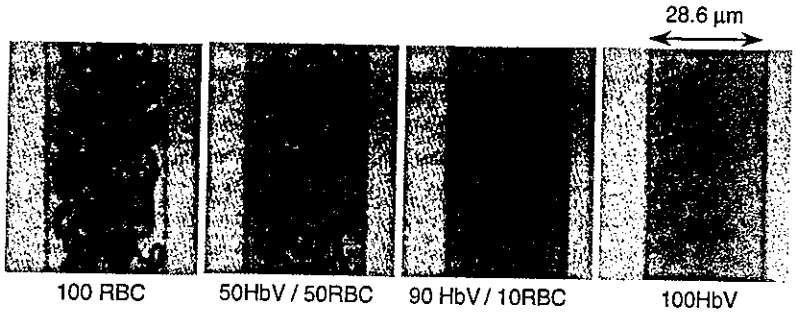

FIG. 9. Flow patterns of the mixture of HbV and RBC suspended in HSA in a narrow tube. HbV particles were homogeneously dispersed in a suspension medium. They tended to distribute in the marginal zone of the flow. The thickness of the RBC-free layer increased with the increasing amount of HbV. The RBC-free phase becomes darker and more semi-transparent, indicating the presence of HbV. Diameter of the tube = 28 μm; [Hb] = 10 g/dl; centerline flow velocity = 1 mm/s

similar with that from RBC alone. On the other hand, the addition of 50 vol% acellular Hb solution to RBC significantly enhanced the rate of deoxygenation. This outstanding difference in the rate of the O_2 release between the HbV suspension and the acellular Hb solution should mainly be due to the difference in the particle size (250 vs. 8 nm) that affects their diffusion for the facilitated O_2 transport. It has been suggested that the faster O_2 unloading from the HBOCs is advantageous for tissue oxygenation [110]. However, this concept is controversial regarding the recent finding that an excess O_2 supply would cause autoregulatory vasoconstriction and microcirculatory disorders [111–113]. We confirmed that HbV does not induce vasoconstriction and hypertension, due to not only the reduced inactivation of nitric oxide as an endothelium-derived vasorelaxation factor, but also possibly the moderate O_2 releasing rate similar to RBC as confirmed in this study.

Effects on Hematological Functions

The biocompatibility of HbV is important to clinical use. Transient thrombocytopenia was one of the most significant hematological effects observed after infusion of liposome-encapsulated Hbs in rodents [114]. Exchange transfusion with unmodified HbV (containing DPPG as a lipid component) in anesthetized rats also resulted in a slightly decreased platelet count, although the change was insignificant [104]. These effects were also observed for administration of negatively charged liposomes [115,116]. The transient reduction in platelet counts caused by liposomes was also associated with

sequestration of platelets in the lung and liver. Platelet activation is necessary to prevent bleeding in vivo; however, nonphysiological activation leads to initiation and modulation of inflammatory responses because platelets contain an array of potent proinflammatory substance. RANTES (Regulated upon activation, normal T-cell expressed and presumably secreted), one of the C-C chemokines, is a useful marker for platelet activation as it is stored in α-granules of platelets and was shown to be released after stimulation. Accordingly, the biocompatibility of HbV was examined by estimating their effects on agonist-induced platelet aggregation response and RANTES release from platelets in vitro [117]. This study on biocompatibility was performed in collaboration with Dr. H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo), and his colleagues.

The effect of low concentration of HbV (Hb: 5.8 mg/dl) on platelet function was assessed by examining an agonist-induced aggregation response, and that of relatively high concentrations of HbV (Hb: 0.29, 1 and 2 g/dl) by measuring the release of RANTES from platelets, which is regarded as a marker of platelet activation. The pre-incubation of platelets with HbV at 5.8 mg/dl of Hb did not affect platelet aggregation induced by collagen, thrombin, and ristocetin. The pretreatment of platelet-rich plasma (PRP) with HbV at concentration up to 2 g/dl of Hb had no aberrant effects on the collagen-induced RANTES release. Furthermore, the collagen-induced release of RANTES from PRP was not affected by longer incubation with HbV at 2 g/dl of Hb. The basal levels of RANTES from PRP were unchanged in the presence of HbV. These results suggest that HbV, at the concentrations studied, have no aberrant effects on platelet functions in the presence of plasma.

The effect of HbV on the coagulation time (PT, APTT) was tested with human plasma. HbV was mixed with human plasma at the ratios of 20%, 40% and 60% v/v. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured automatically. The results were compared with saline and phosphate buffered saline. The PT value increased from 10 s to 15 s with increasing the mixing ratio; however, there was no significant difference between the groups. The APTT value increased from about 40 s to about 50 s with an increase of the mixing ratio; however, there were no significant differences between the groups. The delayed coagulation is due to the dilution of the blood components, and there is no significant effect on the blood coagulation system.

Polymorphonuclear neutrophils (PMNs) are essential cells in the host defense against a variety of infectious agents. Circulating PMNs require activation to migrate to inflammatory sites and then effectively kill pathogens. Previously in the field of drug delivery systems, sterically stabilized liposomes with PEG have been reported to reduce the chemotactic activity of human PMNs in response to zymosan and the bacterially derived peptide, *N*-formyl-

methionyl-leucyl-phenylalanine (fMLP) [118]. Therefore, the effects of the PEG-modified HbV on human PMNs in vitro were studied, focusing on the functional responses to fMLP as an agonist [119]. The pretreatment of PMNs with HbV up to a concentration of 56 mg/dl Hb did not affect the fMLP-triggered chemotactic activity. In parallel to these results, the fMLP-induced upregulation of CD11b (Mac-1) levels on HbV-pretreated PMNs was comparable to that of untreated cells. Furthermore, the pretreatment of PMNs with HbV even at 580 mg/dl Hb did not affect the gelatinase B [Matrix methalloproteinase-9 (MMP-9)] release, suggesting that the fMLP-induced release of secondary and tertiary granules was normal. In addition, the fMLP-triggered superoxide production of PMNs was unchanged by the pretreatment of HbV at 580 mg/dl Hb. Thus, these results suggest that HbV, at the concentrations studied, have no aberrant effects on the fMLP-triggered functions of human PMNs.

Hypertension and Vasoconstriction in Relation with NO and CO

As clinical trials of the chemically modified Hbs are extended to include larger numbers of individuals, it becomes apparent that the principal side effect consistently reported in the administration of acellular Hb solutions is hypertension presumably because of vasoconstriction. Hypertension, a well-defined reaction of the acellular intramolecularly cross-linked Hb (XLHb), was proposed to be beneficial in the treatment of hypotension concomitant to hemorrhagic shock [120]. However, vasoconstriction reduces blood flow, lowering functional capillary density, and therefore affecting tissue perfusion and oxygenation [113,121]. Nitric oxide (NO) scavenging by Hb due to intrinsic high affinity of NO to Hb is the mechanism presumed to cause vasoconstriction and hypertension [122,123]. This theory was validated indirectly using exteriorized rabbit aortic rings in organ baths, where constriction was observed following the addition of acellular Hb solutions as well as an NO synthase inhibitor [124,125]. Different modifications of the Hb molecule cause hypertension that is qualitatively and quantitatively different, and red blood cells (RBCs) and cellular HbV (liposome-encapsulated Hb) do not cause either vasoconstriction or hypertension [99,100,105]. Most evidence for the pressor response is obtained from measurements of systemic pressure, and direct evidence about the mechanism involved is scarce. In previous studies in conscious hamsters fitted with a dorsal skinfold, we found that small arteries of 130–160 μm diameter, termed resistance vessels, exhibit the greatest reactivity in hemorrhagic shock [126], playing a significant role in the regulation of blood flow. Constriction of these resistance vessels in this model was also directly correlated to the pressure response following administration of NO synthase inhibitor [127].

In collaboration with Prof. Intaglietta, we analyzed the relationship between the constriction of resistance vessel and hypertension after administration of acellular Hb and the extent to which the effect is dependent on the size of acellular Hb molecules modified by polymerization, polymer conjugation, and cellular liposome encapsulation [128]. Conscious Syrian golden hamsters with dorsal skinfold preparation were used. After the top load infusion of Hb products (7 ml/kg) into arterial catheters inserted into the jugular vein, mean arterial pressure and heart rate were monitored through the jugular arterial catheter, and microvascular responses were monitored by an intravital microscopy. The Hb products included intra-molecularly crosslinked Hb (XLHb), PEG-conjugated pyridoxalated Hb (PEG-PLP-Hb), hydroxyethyl-starch-conjugated XLHb (HES-XLHb), glutaraldehyde-polymerized XLHb (Poly-XLHb), and HbV. Their molecular diameters were 7, 22, 68, and 224 nm, respectively. The top load infusion of 7 ml/kg of XLHb (5 g/dl) caused the immediate increase of MAP, which was 34 ± 13 mmHg higher 3 h after infusion. There was a simultaneous decrease in the diameter of the resistance vessels (79 ± 8% of basal value) which caused blood flow to decrease throughout the microvascular network. The diameter of smaller arterioles did not change significantly. Infusion of O_2 carriers of greater molecular size resulted in lesser vasoconstriction and hypertension with HbV showing the smallest changes. Infusion of HSA was used as a control and produced no microvascular or systemic effects. Constriction of resistance arteries was found to be correlated to the level of hypertension, and the responses proportional to the molecular dimensions of Hb-based O_2 carriers. Since the results correlate with molecular size it is likely that the effects are related to the diffusion properties of the different Hb molecules.

The liver is a major organ that detoxifies excess amounts of heme by the action of heme oxygenase (HO). HO decomposes protoheme IX to generate biliberdin-IXα and CO. Under normal conditions, the liver contains at least two OH isozymes for physiologic degradation of the heme: HO-1 and HO-2. One of the important roles of the HO reaction is to generate CO that serves as an endogenous regulator that is necessary for maintaining microvascular blood flow [129]. Since Hb strongly binds with CO (about 200 times stronger than O_2), it is necessary to confirm the effects of HbV in hepatic microcirculation in comparison with stroma free Hb solution. Dr. Suematsu et al. studied the perfusion of a rat liver with an acellular Hb solution and HbV, and found out that the Hb solution increased vascular resistance by 30% [130]. The smaller acellular Hb molecules (7 nm) extravasate across the fenestrated endothelium with a pore size of about 100 nm, and reach to the space of Disse. Heme is excessively metabolized by HO-2 to produce CO and bilirubin. Even though CO acts as a vasorelaxation factor in the liver, the excess amount of Hb in the space of Disse rapidly binds CO, resulting in vasoconstriction and

an increase in vascular resistance. On the other hand, HbV (250 nm) is large enough to maintain in the sinusoid, and the vascular resistance is maintained.

These results indicate the importance of the size of the oxygen carriers, and that the size of HbV is appropriate for the maintenance of microvascular blood flow.

Biodistribution and Metabolism of HbV, and Influence on Organ Function

In the physiological condition, free Hb released from RBC is rapidly bound to haptoglobin, and removed from the circulation by hepatocytes. However, when the Hb concentration exceeds the haptoglobin binding capacity, unbound Hb is filtered through the kidney where it is actively absorbed. When the reabsorption capacity of the kidney is exceeded, hemoglobinuria and eventually renal failure occur. The encapsulation of Hb completely suppresses renal excretion, although HbV particles as well as phospholipid vesicles (liposomes) in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [131].

To examine the precise circulation persistence and biodistribution of HbV, we used radiolabelling technique, ^{99m}Tc-labelled HbV, in collaboration with Prof. Phillips at the University of Texas. The HbV co-encapsulated homocysteine (5 mM) was successfully labeled with ^{99m}Tc by using the hexamethylpropylene amine oxime. The circulation half-life of ^{99m}Tc-HbV was determined to be 35 h. In the gamma camera image, the radioactivity in the blood pool of the heart was gradually decreased and those of the liver and spleen were increased with time. The biodistribution data showed the major organs to eliminate the ^{99m}Tc-HbV from the blood circulation were the liver, bone marrow, and spleen, independent of the injection dose. [132].

The influence of HbV on RES, mainly liver and spleen, was studied with carbon clearance measurements and histopathological examination [133]. The HbV suspension was intravenously infused in male Wistar rats (200 g) at dose rates of 10 and 20 ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hs, and at 1, 3, 7 and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the original level at 7 days, and then returned to the original level at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with hematoxylin/eosin, and anti-human Hb antibody staining showed that HbV was metabolized within 7 days. Hemosiderin was slightly confirmed with Berlin blue staining at 3

and 7 days in the liver and spleen, although they disappeared at 14 days, indicating that the heme metabolism, excretion, or recycling of the iron ion proceeded smoothly and siderosis was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the vesicular structure of HbV with a diameter of about 1/40 of RBCs in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Even though infusion of HbV modified the phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs from the histological point of view.

We analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes [134]. The HbV suspension was intravenously infused into male Wistar rats (20 ml/kg). The blood was withdrawn at 8 hs, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver as one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes; however, there were no morphological changes in the pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. In conjunction with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20 ml/kg.

In the series of safety evaluations, the repeated infusion of HbV in Wistar rats was performed at the dose rate of 10 ml/kg/day for 14 days [135]. All the rats tolerated the infusion and body weight increased continuously. The hematological test, serum blood biochemistry, and histopathological examination did not raise any serious concern about the safety of HbV. One day after the final infusion spleen and liver weights increased significantly. Histopathological observation indicated significant HbV accumulation in liver and spleen; however, there was no sign of organ damage. Serum clinical laboratory tests indicated significant increases in lipid components derived provably from HbV particles. After a 2 week interval, spleen and liver weight returned to the original levels; however, a significant amount of hemosiderin was confirmed without serum iron increase. All the concentrations of the lipid

components returned to the original levels. Judging from these results, there was no sign of significant toxicity of HbV at the level of dosage employed.

Summary

The efficacy of HbV as oxygen carriers and their safety have been demonstrated. The advantages of cellular HbV can be summarized as follows:

1. The encapsulated Hb is extremely purified and free from virus, endotoxin, and blood type antigen.
2. There is no chemical modification of Hb. Dissociation of Hb tetramers to dimers is restrained and there is no release of Hb from HbV, preventing renal dysfunction.
3. The oxygen affinity is adequately adjusted and the metHb formation is restrained because both the allosteric effectors and metHb reduction systems can be coencapsulated in the vesicles.
4. HbV can be stored for over 2 years at room temperature, owing to both surface modification with PEG chains and deoxygenation.
5. The surface modification of HbV with PEG chains increases high dispersion stability and is effective to prevent aggregation in blood circulation.
6. The colloid osmotic pressure of the HbV suspension is close to zero. But it is adjustable with the addition of adequate colloids such as HSA, which is important to maintain blood volume. The solution viscosity can be adjusted equivalent to that of blood. This would be important for the shear stress on the vascular wall to regulate vascular tone.
7. HbV suspended in a plasma expander such as HSA and rHSA showed sufficient oxygen transporting capacity comparable with RBC for resuscitation from hemorrhagic shock and extreme hemodilution. It is also applicable for oxygenation of ischemic tissues.
8. The physiological activity of Hb such as binding with NO and CO, production of active oxygen species, heme release, and hemeoxygenase activation, can be minimized by encapsulation. Thus there is less vasoconstriction, hypertension, and oxygen injury.

According to the above achievements, significant efforts have been made to produce HbV with a facility of GMP standard, and to start preclinical and finally clinical trials. The combination of recombinant Hb-vesicles suspended in recombinant albumin would be the most ideal "artificial red blood cells" in the future.

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Physiological Capacity of the Reticuloendothelial System for the Degradation of Hemoglobin Vesicles (Artificial Oxygen Carriers) after Massive Intravenous Doses by Daily Repeated Infusions for 14 Days

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ABSTRACT

A hemoglobin vesicle (HbV; diameter 252 ± 53 nm) or liposome-encapsulated Hb is an artificial oxygen carrier developed for use as a transfusion alternative, and its oxygen-transporting capacity has been well characterized, although critical physiological compartments for the Hb degradation after a massive infusion of HbV and the safety outcome remain unknown. In this study, we aimed to examine the compartments for its degradation by daily repeated infusions (DRI) of HbV, focusing on its influence on the reticuloendothelial system (RES). Male Wistar rats intravenously received the HbV suspension at 10 ml/kg/day for 14 consecutive days. The cumulative infusion volume (140 ml/kg) was equal to 2.5 times the whole blood volume (56 ml/kg). The animals tolerated the DRI well and survived, and body weights continuously increased. One day after DRI, hepatosplenomegaly occurred significantly through the accumulation of large amounts of HbV. Plasma clinical chemistry was overall normal, except for a transient elevation of lipid components derived from HbV. These symptoms subsided 14 days after DRI. Hemosiderin deposition and up-regulation of heme oxygenase-1 coincided in the liver and spleen but were not evident in the parenchyma of these organs. Furthermore, the plasma iron and bilirubin levels remained unchanged, suggesting that the heme-degrading capacity of the RES did not surpass the ability to eliminate bilirubin. In conclusion, phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of senescent RBCs, even at doses greater than putative clinical doses.

Phospholipid vesicles or liposomes have been extensively studied as a carrier of functional (macro)molecules for a drug delivery system, and some are now approved for clinical use as antifungal or anticancer therapies (Lian and Ho, 2001). Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb vesicles (HbV) or liposome-encapsulated Hb, have been developed as artificial oxygen carriers, and their sufficient ability to transport oxygen comparable with blood has been well clarified (Djordjevich et al., 1987; Chang et al., 1992; Izumi et al., 1997; Phillips et al., 1999; Sakai et al., 2004c). The advantages of an artificial oxygen carrier are the absence of blood-type antigens and transfusion-related transmission of infections, and stability during long-term storage. In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion that still has the potential of mismatching, the risk of infections secondary to the infusion of contaminated blood, and the problem of only a few weeks' storage life.

In a series of safety studies of HbV, it has been clarified that the cellular structure and the size of the HbV are advantageous for maintaining a steady blood circulation with-

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ABBREVIATIONS: HbV, Hb vesicle(s); Hct, hematocrit; RES, reticuloendothelial system; PEG, poly(ethylene glycol); RBC, red blood cell; DRI, daily repeated infusion(s); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadexyl-*N*-syccynyl-L-glutamate; MAP, mean arterial pressure; HR, heart rate; UA, uric acid; BUN, urea nitrogen; CRE, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; HO-1, heme oxygenase-1.

out vasoconstriction and hypertension (Sakai et al., 2000a) and that the surface modification of HbV with polyethylene glycol (PEG) is beneficial not only for a longer circulation time (Phillips et al., 1999) but also for suppression of intervesicular aggregation of HbV during preservation for years and in the plasma phase in the peripheral tissues after intravenous infusion (Sakai et al., 1998, 2000b). In our previous report on the histopathological analysis of rats receiving a bolus HbV infusion (20 ml/kg), the HbV particles are recognized as foreign materials and finally captured mainly by the reticuloendothelial system (RES, or mononuclear phagocytic system) in the spleen and liver, and they are promptly degraded (Sakai et al., 2001). These are outstanding characteristics in comparison with molecular Hb that shows a shorter circulation time because it is filtered through the kidneys when the Hb concentration exceeds the haptoglobin concentration and induces hemoglobinuria and eventually renal failure, and it extravasates across the fenestrated endothelium in the liver and induces excess heme catabolism in the hepatocytes and marked sinusoidal constriction (Goda et al., 1998, Kyokane et al., 2001). However, it is not clear whether the physiological capacity of the RES for the degradation and excretion of the components of HbV would be sufficient even after a massive infusion of HbV. The circulation half-life of HbV is within a few days, which is significantly shorter than that of red blood cells (RBCs), and it is anticipated that a massive infusion of HbV would burden the RES and result in abrupt heme degradation and iron overload having the potential to cause deleterious effects.

One of the safety studies of a new drug in the preclinical stage should be a massive dose by daily repeated infusions (DRI) in rodents and nonrodents for at least 14 days at three different dosages; a guideline decided by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. However, the documentation of the DRI studies to the public is scarce, especially in the research field of artificial oxygen carriers (Biro and Greenburg, 1999). In a clinical setting, the amount of an artificial oxygen carrier to be infused should be at least several hundred milliliters, which is significantly greater than the dose of conventional drugs; therefore, it is not clear whether a preclinical protocol for a conventional drug is appropriate for the safety evaluation of artificial oxygen carriers. On the other hand, there may be a need for a repetitive infusion of an oxygen carrier in a clinical situation, such as chronic anemia (Hamilton et al., 2001) or cancer therapy (Teicher et al., 1997). Based on these backgrounds, we tested the DRI of HbV into Wistar rats at one dose rate as a preliminary study to confirm the safety of HbV. Because the dose amount of phospholipid vesicles for use as an oxygen carrier is significantly greater than that used for conventional drugs, the influence of a massive infusion of HbV on the RES and the excretion of the components, especially after heme degradation, are of great concern.

Materials and Methods

Preparation of HbV Suspension. The test fluid, the HbV suspension, was prepared under sterile conditions as reported previously (Sakai et al., 2000b; Sou et al., 2000, 2003). Human Hb was purified from outdated, donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM pyridoxal 5′-phosphate (Aldrich Chemical Co., Milwaukee, WI) as an allosteric effector at a molar ratio of pyridoxal 5′-phosphate/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-*O*-dihexadecyl-*N*-succynyl-L-glutamate (DHSG) at a molar ratio of 5:5:1 (Nippon Fine Chemicals Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Co., Tokyo, Japan). Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DHSG/1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} was 5:5:1:0.033. HbVs were suspended in a physiological salt solution, sterilized using filters (pore size, 0.45 μm, Dismic; Toyo Roshi Co., Tokyo, Japan), and deoxygenated with bubbling N_2 for storage (Sakai et al., 2000b). The physicochemical parameters of the HbV are as follows: particle diameter, 252 ± 53 nm; [Hb], 9.5 g/dl; [metHb], 2.3%; [HbCO], <2%; [lipids], 5.3 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was measured by a modified *Limulus* amebocyte lysate gel-clotting analysis (Wako Pure Chemicals, Tokyo, Japan) and was less than 0.2 endotoxin unit/ml (Sakai et al., 2004a).

In our previous reports on resuscitation from hemorrhagic shock or extreme hemodilution, the HbV was suspended in a 5-g/dl albumin solution as a plasma expander to regulate the colloid osmotic pressure to 20 mm Hg (Sakai et al., 2004c). However, it is anticipated that the DRI of HbV suspended in albumin would result in enhanced hypervolemia. Because the main purpose of this DRI study was to clarify the safety of HbV and not albumin, HbV was simply suspended in a physiological saline solution.

Daily Repeated Infusion of HbV. All animal studies were approved by the Animal Subject Committee of the Keio University School of Medicine and performed according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 rev. 1985).

The experiments were carried out using 34 male Wistar rats (145 ± 4 g; Saitama Experimental Animals, Kawagoe, Japan). All the rats were housed in cages and provided with food and water ad libitum in a temperature-controlled room on a 12-h dark/light cycle. At first, the rats were anesthetized lightly with diethyl ether inhalation and then 1.5% sevoflurene (Maruishi Pharmaceutical Co., Osaka, Japan) using a vaporizer (model TK-4 Biomachinery; Kimura Medical, Tokyo, Japan) to immobilize them for every infusion. Every day for 14 days, the rats received HbV (n = 12) or saline (n = 12) via the tail vein using an indwelling needle (24-gauge; Nipro Co., Osaka, Japan) at a dose rate of 10 ml/kg with an injection rate of 1 ml/min. The total volume of the infused HbV into a rat for 14 days reached 140 ml/kg, which was equal to 2.5 times the actual blood volume of the rat (56 ml/kg). The infused total solid material (Hb and lipids) is calculated to be 20,689 mg/kg (1478 mg/kg/day × 14 days). The rats were weighed every day just before every infusion to calculate the amount of the infusion. After every infusion, the needle was immediately removed and the bleeding was stopped by applying pressure for a short time. The two groups (n = 12) were divided in half (n = 6 × 2), and six rats were sacrificed 1 day after the final 14th infusion. The remaining six rats were sacrificed at 14 days after the final infusion. Ten animals without the infusions were used to obtain control values.

Hematological Test. A hematological examination was performed at 1, 3, 7, 9, and 12 days during the DRI and at 1, 7, and 14 days after the final infusion. About 200 μl of blood was collected from a tail vein when an indwelling needle was inserted for HbV infusion. Seventy microliters was immediately diluted with 200 μl of citrate solution for a blood cell counter (Sysmex KX-21, Kobe, Japan), and the rest of the blood was inserted into a glass capillary (Terumo Co., Tokyo, Japan) for hematocrit (Hct) measurements. In this study, Hct indicates the volume of RBC and does not include the volume of HbV. The concentration of HbV in the plasma was measured by a cyanomethemoglobin method.

Hemodynamic and Blood Gas Parameters, Blood Glucose Level, and Urinalysis. One day or 2 weeks after the final infusion, the rats were anesthetized with 1.5% sevoflurene inhalation. A polyethylene tube (PE-50; Natsume Co., Tokyo, Japan) was inserted into the carotid artery for measurement of the mean arterial pressure (MAP), the heart rate (HR) by a recording system (Polygraph system 1000; Nippon Koden, Tokyo, Japan) and for withdrawing blood for various measurements. For the blood gas analysis, blood samples were collected in 70 IU/ml heparinized microtubes (125 μl, Clinitubes; Radiometer Nederland, Copenhagen, Denmark) and injected into a pH/blood gas analyzer (model ABL 555; Radiometer Nederland) for analyses of the arterial blood O_2 tension, arterial blood carbon dioxide tension, pH, base excess, and lactate. The blood glucose level was measured with a Medisafe Reader (GR-101; Terumo Co., Tokyo, Japan). Urinalysis was performed by dip-stick-testing (UA-L08M; Terumo Co.) as a qualitative measurement. A urine specimen of a rat was collected in a transparent plastic bag when the rat was lightly anesthetized with diethyl ether, and a test stick was dipped in the collected urine. In each item, the levels were judged by visual examination of the color identification after a specific time of exposure according to the instructions, in the order of protein (10 s), pH (10 s), occult blood (20 s), ketone body (20 s), urobilinogen (20 s), glucose (30 s), nitrite (30 s), and bilirubin (40 s).

Plasma Clinical Chemistry. A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma that was turbid and red/brown colored due to the presence of PEG-modified HbV particles, especially in the samples taken one day after DRI. The plasma was ultracentrifuged (50,000*g*; 20 min) to remove the HbV particles (Sakai et al., 2003). The obtained transparent plasma specimens were stored in a freezer at −80°C until the clinical chemistry tests (BML, Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, cholinesterase, leucine amino peptidase, creatine phosphokinase, amylase, lipase, aldosterone, total cholesterol, cholesterol ester, free cholesterol, HDL-cholesterol, β-lipoprotein, triglyceride, free fatty acid, phospholipids, total lipids, uric acid (UA), urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate, unsaturated iron binding capacity, and Fe^{3+}. All the analytical methods were described in our previous articles (Sakai et al., 2003, 2004b).

Blood Coagulation Test and Fibrinogen Concentration. For the blood coagulation test at 1 and 14 days after the final infusion of HbV or saline, 1.8 ml of the withdrawn blood was immediately mixed with 0.2 ml of 3.8% sodium citrate solution. The plasma fraction, obtained by gentle centrifugation, was analyzed for prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (BML).

Histopathological Examination. The animals were finally laparotomized and sacrificed by acute bleeding from the abdominal aorta, and the liver, spleen, and kidney were resected for weight measurements and also all the other organs were obtained for a histopathological study. They were fixed in 10% buffered formalin (Wako Pure Chemicals) immediately after removal, and the paraffin sections were stained with hematoxylin & eosin, and Berlin blue.

Immunohistochemistry was performed to detect rat heme oxygenase-1 (HO-1) and human Hb from the injected HbV in the rat spleen and liver. Four-micrometer-thick paraffin sections were mounted on 3-aminopropyl triethoxysilane-coated glasses. The sections were treated with 0.03% H_2O_2 in methanol for 10 min at room temperature to block the endogenous peroxidase activity. For antigen retrieval, the sections were also treated with proteinase K (0.4 mg/ml; DakoCytomation California Inc., Carpinteria, CA) for 10 min at room temperature. After blocking the nonspecific binding with 5% normal goat serum, they were incubated with mouse monoclonal antibody against rat HO-1 (20 μg/ml; GTS-3, TaKaRa, Tokyo, Japan) at 4°C overnight. They were then incubated for 30 min at room temperature with goat antibodies against mouse immunoglobulins conjugated to the amino acid polymer [no dilution; Histofine Simple Stain MAX-PO(M), Nichirei Co., Tokyo, Japan]. Color was developed with 3,3'-diaminobenzidine tetrahydrochloride (0.2 mg/ml; Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl, pH 7.4, containing 0.003% hydrogen peroxide. Subsequently, the sections were treated with 5% normal swine serum for 30 min at room temperature and reacted with rabbit polyclonal antibodies against human Hb (1:500 dilution; DakoCytomation A/S, Glostrup, Denmark) for 60 min at room temperature. They were further incubated with alkaline phosphatase-conjugated swine antibodies against rabbit immunoglobulins (1:100 dilution; DakoCytomation A/S). Color development was performed using a New Fuchsin Substrate kit (Nichirei Co.), and the sections were counterstained with hematoxylin.

Data Analysis. Differences between the control and the treatment group were analyzed using a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered statistically significant if $p < 0.05$. All the data are shown as mean ± S.D. For the results of the plasma clinical chemistry, the allowance of twice the standard deviation (2× S.D.) of the baseline values is indicated in the figures in considering the variable nature of these parameters.

Results

Body Weight. The body weight of rats in the HbV group (baseline, 144 ± 3 g) showed a monotonous increase during the 14 days of the DRI period and reached 195 ± 12 g (Fig. 1); however, this was slightly but significantly suppressed ($p < 0.05$) in comparison with the control saline group (220 ± 13 g). The body weight in the HbV group increased to 265 ± 14 g at 14 days after DRI. No significant difference was noted in the body weight compared with that of the saline control group (280 ± 22 g).

Hematological Changes and Concentration of HbV in Blood. The Hct of the HbV group (baseline, 41.7 ± 2.1%) tended to decrease to 37.5 ± 0.9% 1 day after DRI, which was lower than that of the saline group (44.7 ± 2.0%) (Fig. 2). However, after 14 days, the Hct of the HbV group increased to 45.1 ± 1.9%, which was comparable with that of the saline group (47.8 ± 2.7%). The numbers of white blood cells and platelets were comparable with those of the saline control group throughout the observation period.

The concentration of HbV immediately after every infusion was estimated from the volumes of the whole blood (56 ml/kg) and the infused volume of HbV (10 ml/kg) and was plotted

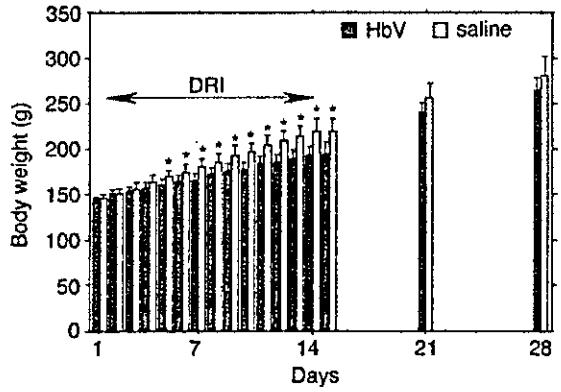

Fig. 1. Time course of the gain in body weight during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. Both groups showed monotonous increases; however, after the 5th day, a significant difference was observed. Seven days after the final infusion, there were no significant differences between the two groups. The values are mean ± S.D. *, significantly different between the groups ($p < 0.05$).

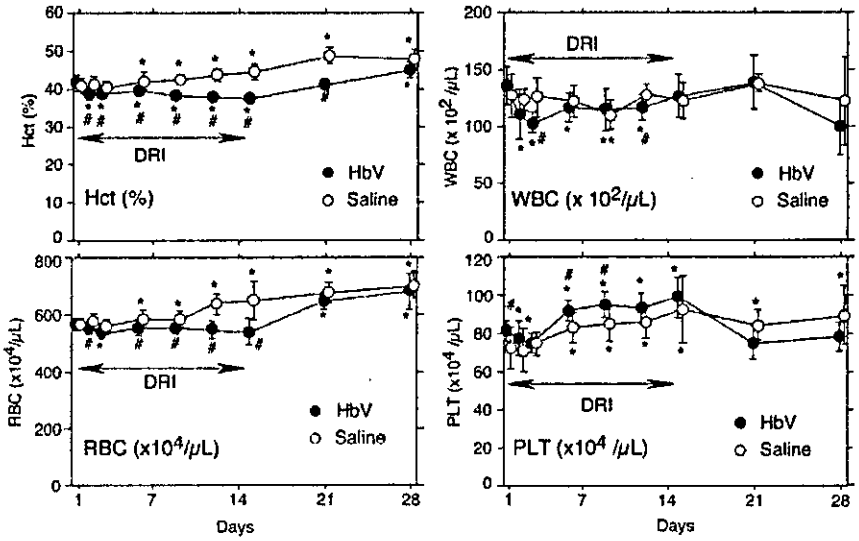

Fig. 2. Time course of Hct and blood cell counts during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. #, significantly different between the groups ($p < 0.05$). *, significantly different versus the baseline values ($p < 0.05$).

with open circles, and the measured concentration of HbV just before every infusion was plotted with solid circles (Fig. 3). The concentration of HbV just after the first infusion was estimated to be about 3 g/dl, and 1 day later, it decreased to 1.3 g/dl, with a half-life of about 22 h. The half-life of the second infusion seemed to become shorter. The DRI resulted in the accumulation of HbV in the plasma and it increased to 5.2 g/dl 1 day after the 14th infusion. However, 1 week after the final infusion, no HbV was confirmed in the plasma, indicating that all of the HbV was captured by the RES.

Coagulation Test. A coagulation test indicated that the HbV group showed a slight prolongation in APTT from 24.3 ± 1.3 s at the baseline to 36.0 ± 11.8 s 1 day after the DRI but changed to 40.3 ± 20.3 s after 14 days with marked individual variations. On the other hand, there was no noticeable change in the PT for the HbV group (Fig. 4). The fibrinogen concentration (baseline, 223 ± 12 mg/dl) significantly increased for all groups (HbV group, 1 day after DRI,

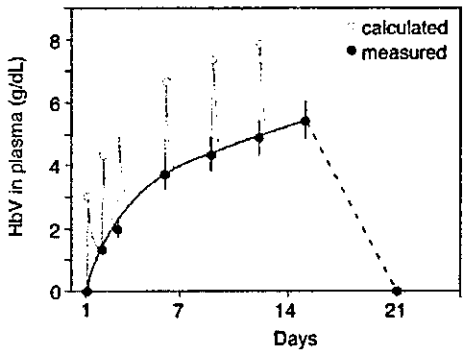

Fig. 3. Time course of the HbV concentration in the plasma phase during and after DRI of HbV for 14 days at a dose rate of 10 ml/kg/day. It is assumed that immediately after the first infusion, the concentration should be around 3 g/dl, and 1 day later, it decreased to about 1.2 g/dl. Immediately after the second infusion, the concentration should be about 4.3 g/dl and decreased to 2 g/dl 1 day later. The half-life of the HbV apparently decreases with multiple infusions. The concentration tended to reach a plateau. The final HbV concentration reached 5.3 g/dl, and this completely disappeared 7 days after DRI.

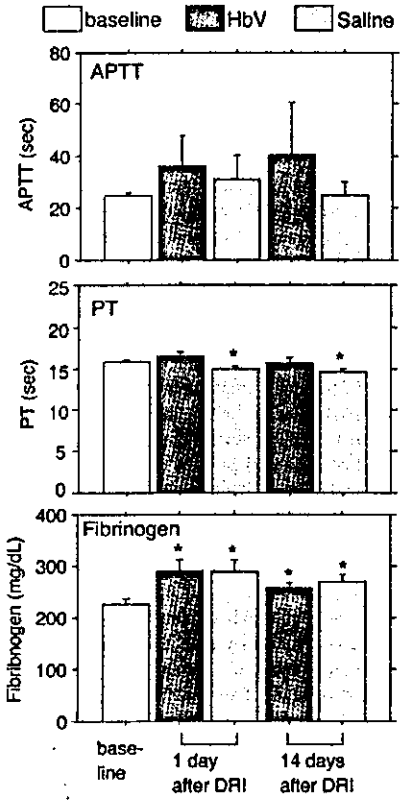

Fig. 4. Parameters for blood coagulation, APTT and PT, and fibrinogen concentration after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

289 ± 22 mg/dl; 14 days after DRI, 255 ± 11 mg/dl), probably due to the stress of infusion and influence on the liver function.

Blood Pressure, Heart Rate, and Blood Gas Parameters. Table 1 summarizes the blood gas parameters, blood glucose level, MAP, and HR 1 day after the DRI of HbV. There is no abnormal value except for an increase in MAP of

TABLE 1

Blood gas parameters, blood glucose level, MAP, and HR 1 and 14 days after DRI of HbV

Baseline values are also listed. The values are mean ± SD.

Parameters	1 Day after DRI	14 Days after DRI	Baseline
pH	7.44 ± 0.03	7.43 ± 0.03	7.48 ± 0.30
$PaCO_2$ (torr)	41 ± 3	44 ± 4	37 ± 4
PaO_2 (torr)	76 ± 4	75 ± 7	82 ± 7
Lactate (mM)	0.93 ± 0.12	0.88 ± 0.38	1.59 ± 0.45
Base excess (mM)	3.1 ± 1.3	4.0 ± 0.8	4.5 ± 1.3
Glucose (mg/dl)	147 ± 24	127 ± 9	146 ± 10
MAP (mm Hg)	125 ± 4*	111 ± 12	101 ± 8
HR (beats/min)	419 ± 10	402 ± 33	404 ± 42

* Significantly different versus the baseline group ($p < 0.05$).

the HbV group 1 day after DRI (125 ± 4 mm Hg) in comparison with the baseline value (101 ± 8 mm Hg).

Urinalysis. The color of the urine was normal for all groups, and there was no sign of hemoglobinurea. Table 2 summarizes the results of the urinalysis. The HbV group showed a slight increase in the protein concentration. There were no significant signs of any organ damage. Urobilinogen and bilirubin were within the normal range in spite of the large amount of HbV infusion.

Organ Weights. The liver and spleen are thought to be the main organs that trap and degrade HbV. As shown in Fig. 5, significant splenomegaly and hepatomegaly were confirmed 1 day after DRI. The percentage of spleen weight relative to the body weight increased from 0.33 ± 0.04% at the baseline to 0.94 ± 0.07 1 day after DRI, about 2.9 times the baseline value. This returned to 0.41 ± 0.03% after 14 days. The percentage of liver weight relative to the body weight increased from 4.81 ± 0.15% at the baseline to 5.83 ± 0.37% 1 day after DRI; and it returned to 4.33 ± 0.20%, comparable with the baseline after 14 days. The color of the liver was darkened just after DRI, however, it returned to its normal color 14 days after DRI. The kidney weight did not show any significant increase but tended to show a slight decrease for all groups.

Plasma Clinical Chemistry. A significant amount of the HbV particles was present in the plasma one day after DRI. However, they could be easily removed from the plasma by ultracentrifugation (50,000*g*; 20 min) (Sakai et al., 2003), and we could avoid any interference effect of HbV in the colorimetric and turbidimetric analyses in the plasma clinical chemistry. The parameters affecting the liver function (total

TABLE 2

The results of urinalysis for the HbV and saline control groups 1 and 14 days after DRI ($n = 6$)

The numbers indicate the counts of rats. Interpretation of judgment for the reading levels: protein (mg/dl), − (negative), ± (15), + (30), ++ (100), +++ (250), and ++++ (1000); occult blood (counts/μl), − (negative), + (10), ++ (50), and +++ (250); ketone body (mg/dl), − (negative), + (10), ++ (50), and +++ (100); urobilinogen (mg/dl), − (negative), ± (0.5), + (2), ++ (4), and +++ (8); glucose (mg/dl), − (negative), ± (50), + (150), ++ (500), and +++ (2000); nitrite, − (negative), and + (0.03–0.2 mg/dl); and bilirubin (mg/dl), − (negative), + (0.5), ++ (1), and +++ (2.5).

Parameters	Levels	1 Day after DRI		14 Days after DRI		Baseline
		HbV	Saline	HbV	Saline[a]	
Protein	−	0	1	2	0	2
	±	4	5	1	2	4
	+	2	0	2	2	0
	++	0	0	1	1	0
	+++	0	0	0	0	0
	++++	0	0	0	0	0
pH	5	0	2	0	0	0
	6	6	2	2	2	0
	7	0	1	4	2	5
	8	0	1	0	1	1
	9	0	0	0	0	0
Occult blood	−	3	2	4	3	4
	+	3	4	2	1	1
	++	0	0	0	1	1
	+++	0	0	0	0	0
Ketone body	−	0	0	0	0	0
	+	6	6	5	5	6
	++	0	0	1	0	0
	+++	0	0	0	0	0
Urobilinogen	−	0	0	0	0	0
	±	6	6	6	5	6
	+	0	0	0	0	0
	++	0	0	0	0	0
Glucose	−	6	6	6	5	6
	±	0	0	0	0	0
	+	0	0	0	0	0
	++	0	0	0	0	0
Nitrite	−	6	6	6	5	6
	+	0	0	0	0	0
Bilirubin	−	6	6	6	5	5
	±	0	0	0	0	1
	+	0	0	0	0	0
	++	0	0	0	0	0
	+++	0	0	0	0	0

[a] $n = 5$.

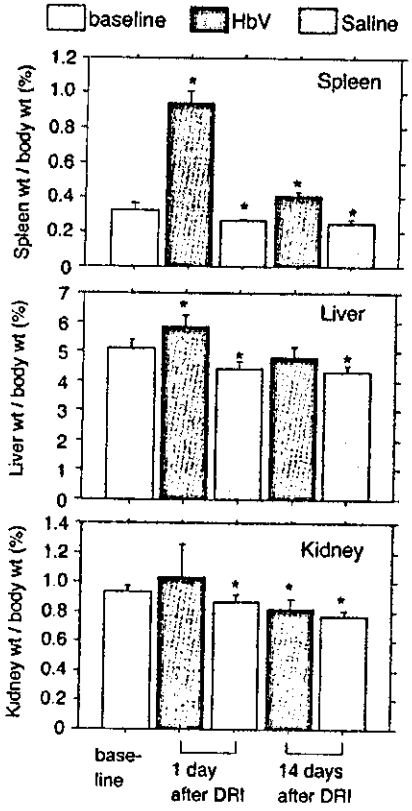

Fig. 5. Changes in organ weights (spleen, liver, and kidney) after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

protein, albumin, aspartate aminotransferase, alanine aminotransferase, leucine amino peptidase, alkaline phosphatase, γ-glutamyltransferase, and cholinesterase) did not show any noteworthy changes in the HbV group (Fig. 6).

The parameters affecting the function of the kidneys (CRE, uric acid, and BUN) varied within the normal ranges. The parameter reflecting the heart and skeletal muscle, creatine phosphokinase, did not show any noticeable change. Aldosterone, released from adrenal gland to regulate ionic balance, did not show noticeable change. A significant change was observed for the parameters of pancreatic function. In the HbV group, the lipase activity (baseline, 8.5 ± 1.4 U/l) increased to 48.5 ± 16.8 U/l 1 day after DRI and tended to decrease to 33.2 ± 29.4 U/l after 14 days. On the contrary, amylase activity (baseline, 1613 ± 74 U/l) did not show an increase but a slight decrease to 1455 ± 28 U/l 1 day after DRI and returned to 1546 ± 77 U/l after 14 days.

The concentrations of the cholesterol components (total and free cholesterols, and cholesterol ester) and lipids (β-lipoprotein, total lipids, and phospholipids) significantly increased 1 day after the final infusion (Fig. 7). For example, total cholesterol (baseline, 72.6 ± 7.5 mg/dl) increased to 182.2 ± 22.6 mg/dl after DRI. However, they returned to the original values 14 days after DRI. These increases should indicate that cholesterol and phospholipid (probably DPPC) are released from the RES after entrapping the HbV particles. Bilirubin and ferric iron, which should be released from the Hb decomposition, were minimal. Unsaturated iron binding capacity did not show noticeable changes. The electrolyte concentrations varied within the normal range.

Histopathological Examination. Histopathological examination 1 day after DRI showed a significant amount of HbV accumulated in the red pulp zone in the spleen, and very few RBCs were seen (Fig. 8a). In the liver, the presence of Kupffer cells that captured a large amount of HbV was seen (Fig. 8b). In the kidneys, the mesangial cells in the renal glomerulus seemed to entrap HbV (Fig. 8c). These organs and the adrenal gland were slightly stained with Berlin blue (data not shown), indicating that the decomposition of heme should have already started. No morphological change was noted in the myocardium; however, some slightly stained particles were observed (Fig. 8d). The pancreas (Fig. 8e), lungs (Fig. 8f), intestine, stomach, brain, thymus, testis, and skin did not show significant abnormalities.

Fourteen days after DRI, the images of the accumulated HbV almost disappeared in all organs. However, there were materials that were moderately stained with Berlin blue in the red pulp zone of the spleen (Fig. 9a), liver (Fig. 9b), bone marrow (Fig. 9c), and slightly in the kidney (Fig. 9d) and adrenal gland (Fig. 9e)

Immunohistochemical analysis of the liver and the spleen clarified the presence of human Hb in HbV as pink-colored areas that were stained with anti-human Hb-antibody 1 day after DRI (Fig. 10, a and b). In the spleen, the presence of HO-1 was confirmed as brown-colored stains in the cytoplasm of the macrophages in the red pulp zone both at 1 and 14 days after DRI (Fig. 10, a and c). In the liver, the presence of HO-1 was confirmed in the Kupffer cells only at 14 days after DRI (Fig. 10d). No HO-1 was confirmed in the parenchyma of these organs.

Discussion

Our primary finding is that all the rats tolerated the DRI of HbV well for 14 days with no deteriorative signs in organ functions, due to the preferable effect of Hb encapsulation in phospholipid vesicles that minimizes the toxicity of molecular Hbs and delivers them to the RES as a physiological compartment for degradation and detoxification of foreign materials. The RES had sufficient capacity for the degradation of HbV, even though the total infused volume reached 140 ml/kg, which was equal to 2.5 times the actual blood volume of the rat (56 ml/kg) and was significantly larger than the dose of multiple infusions of liposomes for antifungal and antitumor targeting (Fielding et al., 1999; Charrois and Allen, 2003).

The body weight of the HbV group monotonously increased, whereas the rate was slightly slower than that of the saline control group. It is speculated that the infusion of HbV, which could not be excreted easily in the urine and remain in circulation, could 1) disturb physiological functions and suppress the growth of the animals, 2) put the animals under stress and reduce their appetite, or 3) tend to accelerate the catabolism. In spite of such a condition, the components of HbV could be used as a part of the cellular components for the growth of rats. One and 2 weeks after DRI, there was no significant difference in the body weight between the groups.

The numbers of RBCs, whole blood cells, and platelets showed moderate changes, even though there were some

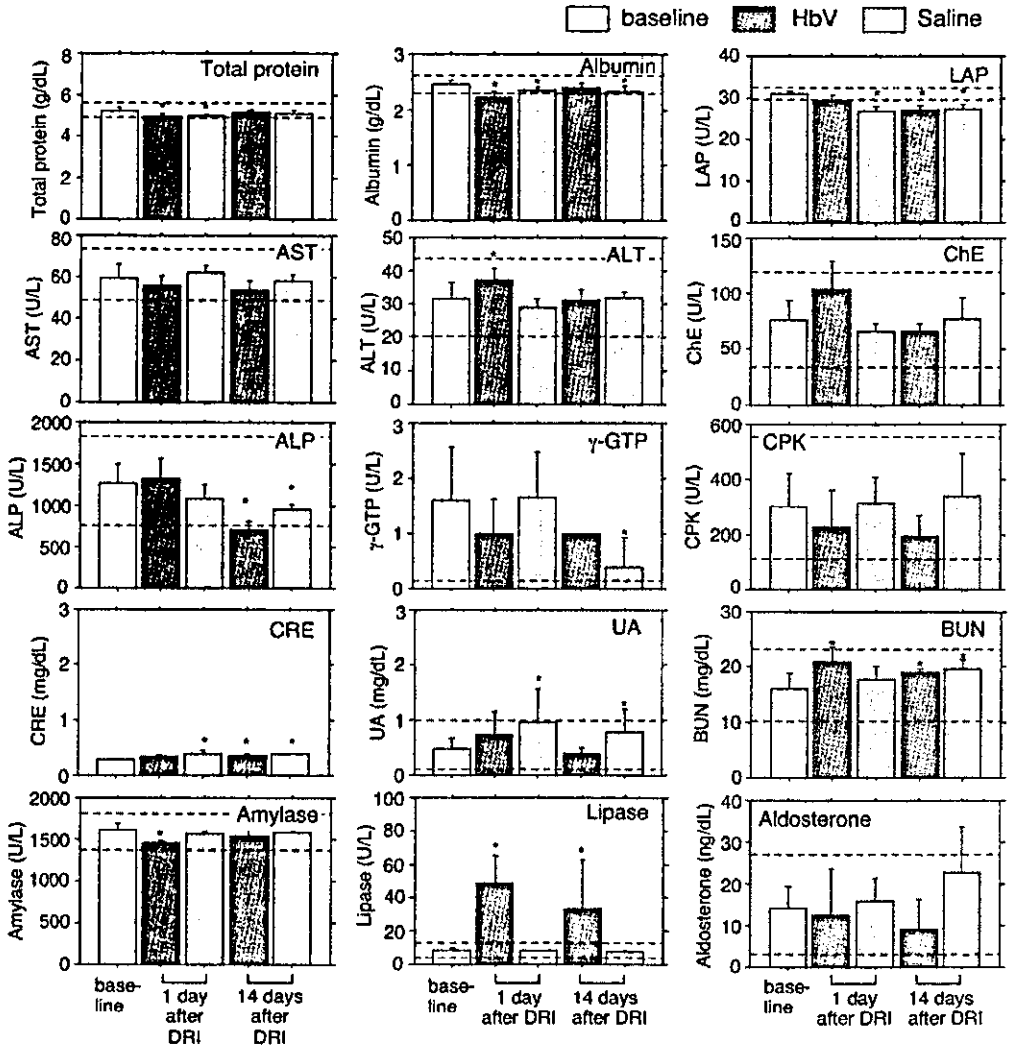

Fig. 6. Plasma clinical chemistry tests reflecting the organ functions such as liver, pancreas, and kidneys and the metabolism of Hb after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$). The dotted lines indicate the levels of 2× S.D. LAP, leucin amino peptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ChE, cholinesterase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase; CPK, creatine phosphokinase; CRE, creatinine; UA, uric acid; BUN, urea nitrogen.

significant differences between the HbV and the saline groups. Hct and RBC counts decreased significantly for the HbV group, probably due to the dilution of blood by hypervolemia, or suppression of erythropoiesis (release of erythropoietin) because the renal cortex would be exposed to the increased oxygen content in the blood during DRI of HbV as oxygen carriers. The slight hypertension 1 day after DRI would be related to the blood hyperviscosity or hypervolemia due to the presence of HbV. However, the Hct and RBC counts returned to levels similar to those of the saline group 14 days after DRI. The time course of the HbV concentration in plasma indicates that the rate of HbV clearance gradually increased and the concentration reached a plateau, probably due to the nonspecific phagocytic activation of the RES that was clarified previously by a carbon clearance measurement (Sakai et al., 2001). The accelerated liposome clearance of the second infusion was well characterized (Claassen et al., 1988; Laverman et al., 2001); however, its mechanism, antibody formation or complement activation is controversial (Dams et al., 2000; Ishida et al., 2003).

In our previous report, the bolus HbV infusion (20 ml/kg) resulted in significant splenomegaly (about 100% increase) and hepatomegaly (13%) (Sakai et al., 2004b). In the present DRI study, splenomegaly was enhanced (190%), whereas hepatomegaly was similar (14%), indicating that the spleen had a larger capacity for HbV clearance. A large amount of HbV accumulated in the red pulp zone of the spleen and in Kupffer cells of the liver; however, 14 days later it disappeared and the splenohepatomegaly completely subsided. The spleen and the liver showed significant hemosiderin deposition; however, the enzyme concentrations that reflect the liver function did not show any abnormal values.

One day after DRI, the mesangial cells in the renal glomerulus seemed to entrap HbV in their intracellular spaces, and the same portion was stained with Berlin blue 1 and 14 days after DRI. In our previous report on the bolus HbV infusion, there was no abnormality in the kidneys (Sakai et al., 2004b). According to Rudolph et al. (1995), liposome-encapsulated Hb without PEG-modification aggregated in the plasma and showed a slight accumulation in the kidneys. Even though our PEG-modified HbV does not induce intervesicular aggregation, HbV would tend to be aggregated during the longer circulation time due to the DRI. No abnormal value was noted for UA, BUN, and CRE, although urinalysis showed a slight increase in protein levels.

Lipase activity, but not that of amylase, significantly in-

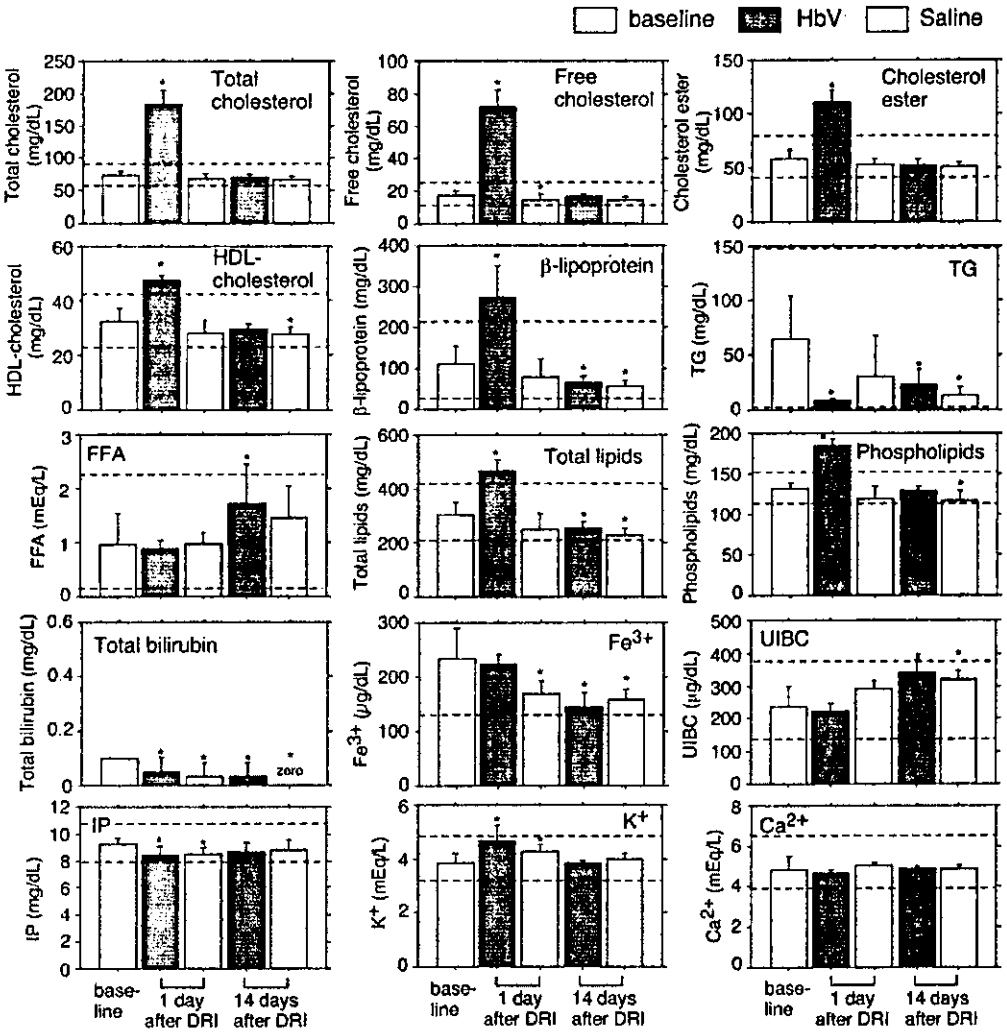

Fig. 7. Plasma clinical chemistry tests reflecting the metabolism of lipids and Hb and electrolytes 1 or 14 days after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group. The dotted lines indicate the levels of 2× S.D. TG, triglyceride; FFA, free fatty acid; UIBC, unsaturated iron-binding capacity; IP, inorganic phosphate.

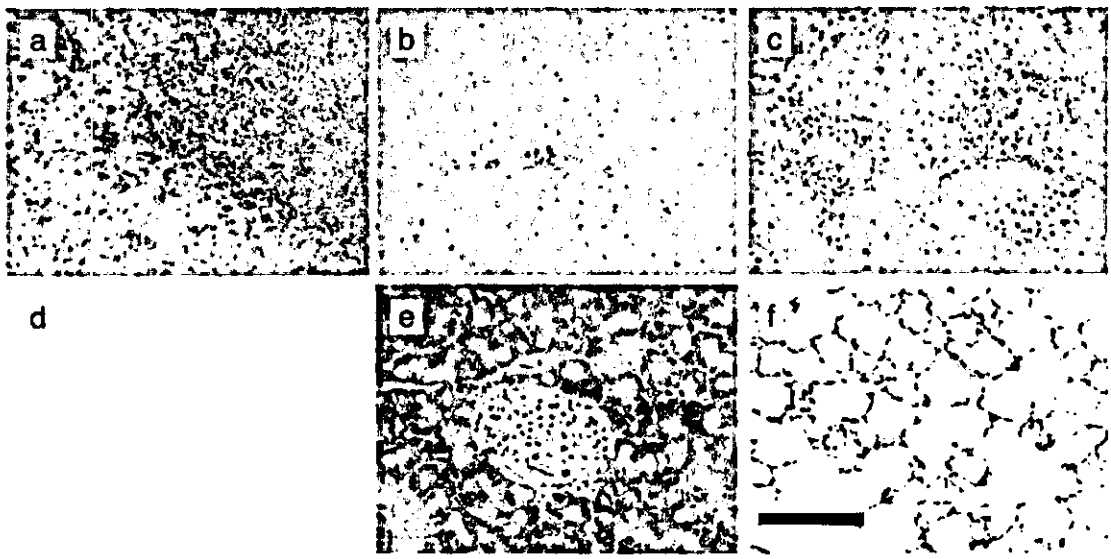

Fig. 8. Histology of spleen (a), liver (b), kidneys (c), heart (d), pancreas (e), and lungs (f) 1 day after DRI of HbV. A significant amount of HbV was accumulated in the red pulp zone of the spleen. The invasion of a significant number of Kupffer cells with HbV was seen in the liver. In the kidneys, the mesangial cells in the renal glomeruli seemed to entrap HbV. The myocardium showed slight staining with Berlin blue. No significant pathological changes are noted in the pancreas and lungs. Scale bar, 100 μm. Hematoxylin and eosin stains (a, b, c, e, and f) and Berlin blue stain (d).

Fig. 9. Histology of spleen (a), liver (b), bone marrow (c), kidneys (d), and adrenal gland (e) 14 days after DRI. Berlin blue staining was performed to examine the presence of hemosiderin. Scale bar, 100 μm.

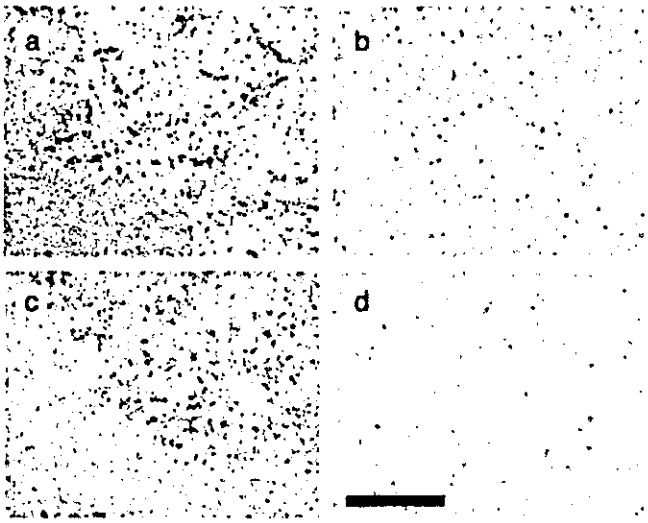

Fig. 10. Double immunohistochemical staining for HO-1 and human Hb in HbV in the rat spleen (a and c) and liver (b and d), 1 (a and b) and 14 days (c and d) after DRI of HbV. The tissues were stained with anti-rat HO-1 monoclonal antibody (GTS-3). The brown-colored portions (a, c, and d) indicate the presence of HO-1, and the pink or gray-beige areas (a and b) indicate the presence of a large amount of HbV. Scale bar, 100 μm.

creased in the HbV group, whereas there was no histopathological abnormality in the pancreas. A similar tendency was observed after the bolus HbV infusion (20 ml/kg) (Sakai et al., 2004b). This level of increment was significantly smaller than the value for the Wistar rats with acute necrotizing pancreatitis that increased the lipase activity from 10 to 475 to 5430 IU/l (Hofbauer et al., 1996). One possible reason for the moderate and specific increase in lipase activity would be related to the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes (Stuecklin-Utsch et al., 2002), because pancreatic lipase hydrolyzes not only triglyceride but also phosphatidylcholine (Rowland and Woodley, 1980). However, the mechanism is not clear, and the pancreatic function should be carefully monitored in the ongoing safety studies.

The plasma lipid components significantly increased after the DRI of HbV. They should be derived from HbV because it contains a large amount of cholesterol and DPPC, and they would be liberated after the HbV particles are captured and degraded in the RES. It is reported that once liposome is captured in the Kupffer cells, the diacylphosphatidylcholine is metabolized and is reused as a cell membrane component or excreted in the bile (Dijkstra et al., 1985; Verkade et al., 1991). Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV (diameter, 250 nm) cannot diffuse across the fenestrated endothelium into the space of Disse (Goda et al., 1998). Cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al., 1986). Judging from the results showing that the increases in the plasma lipid components were transient, the lipid components of HbV would gradually be redistributed, metabolized, and excreted in the same manner within 14 days after DRI. However, the details have to be confirmed by the biodistribution of the radiolabeled components.

In spite of the massive HbV infusions, the plasma bilirubin and iron levels did not increase. Urinalysis also showed no increase in the urobilinogen and bilirubin. The anti-human Hb antibody staining detected temporal distributions of HbV in the spleen and liver. The excess amount of heme from Hb in HbV should be metabolized by the inducible form of HO-1 in the spleen macrophages and the liver Kupffer cells, as shown in Fig. 10 (Braggins et al., 1986; Goda et al., 1998). Bilirubin should be excreted in the bile as a normal physiological pathway even during the massive doses of HbV. No increase in the plasma bilirubin level indicated that there was no obstruction or stasis of bile in the biliary tree and that the heme-degrading capacity of the RES did not surpass the ability to eliminate

bilirubin. Berlin blue staining revealed the presence of hemosiderin in the liver, spleen, kidneys, adrenal gland, and bone marrow 14 days after DRI and also in the myocardium 1 day after DRI. Both ferritin and hemosiderein store and release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert (O'Connell et al., 1989). Multiple blood transfusions often induce hemosiderosis in many organs. Accordingly, Hb encapsulation in the phospholipid vesicles would guarantee the smooth metabolic route of HbV that is similar to the well characterized metabolic route of senescent RBCs in the liver Kupffer cells and spleen macrophages (Bennett and Kay, 1981; Hirano et al., 2001). This would be a great advantage over molecular Hb that incurs not only filtration across the fenestrated endothelium of the glomerular capillary in the kidneys resulting in shorter circulation time and renal failure but also extravasation from the sinusoidal caliber in the liver, causing cancellation of the CO-mediated fail-safe mechanism for conserving sinusoidal patency and bile formation (Kyokane et al., 2001).

In conclusion, all the rats tolerated the DRI of HbV with no deteriorative signs of the organ functions. The phospholipid vesicles for Hb encapsulation would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of not only foreign materials but also the senescent RBCs. However, it has to be considered that in humans the circulation time of HbV and its degradation rate in the RES would be different compared with those in rats, because the circulation time of stealth liposomes and the life span of RBCs are different between rodents and humans (Landaw, 1988; Gabizon et al., 2003). A shock condition may also influence on the RES function.

Our results would provide important information not only for the ongoing safety studies of HbV but also for the overall research on liposomal drugs, because this study is the first attempt to infuse repetitively such a large amount of phospholipid vesicles.

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