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 (50,000g; 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

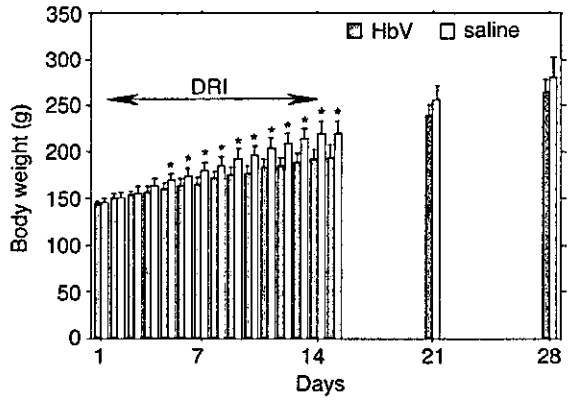

Fig. 1. Time course of the gain in body weight during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. Both groups showed monotonous increases; however, after the 5th day, a significant difference was observed. Seven days after the final infusion, there were no significant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

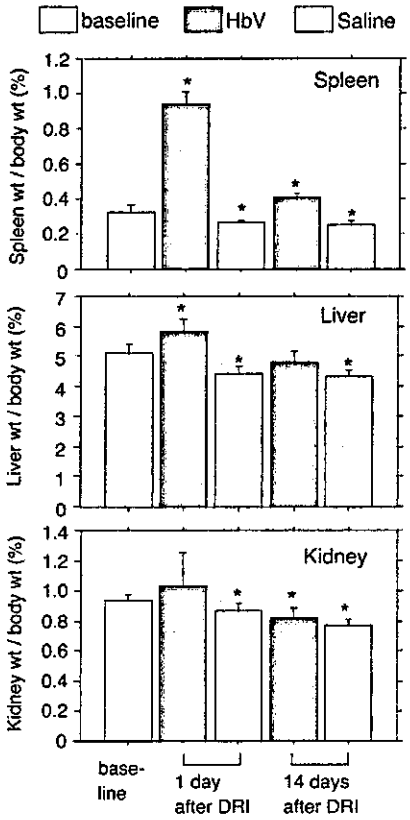

Fig. 5. Changes in organ weights (spleen, liver, and kidney) after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

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

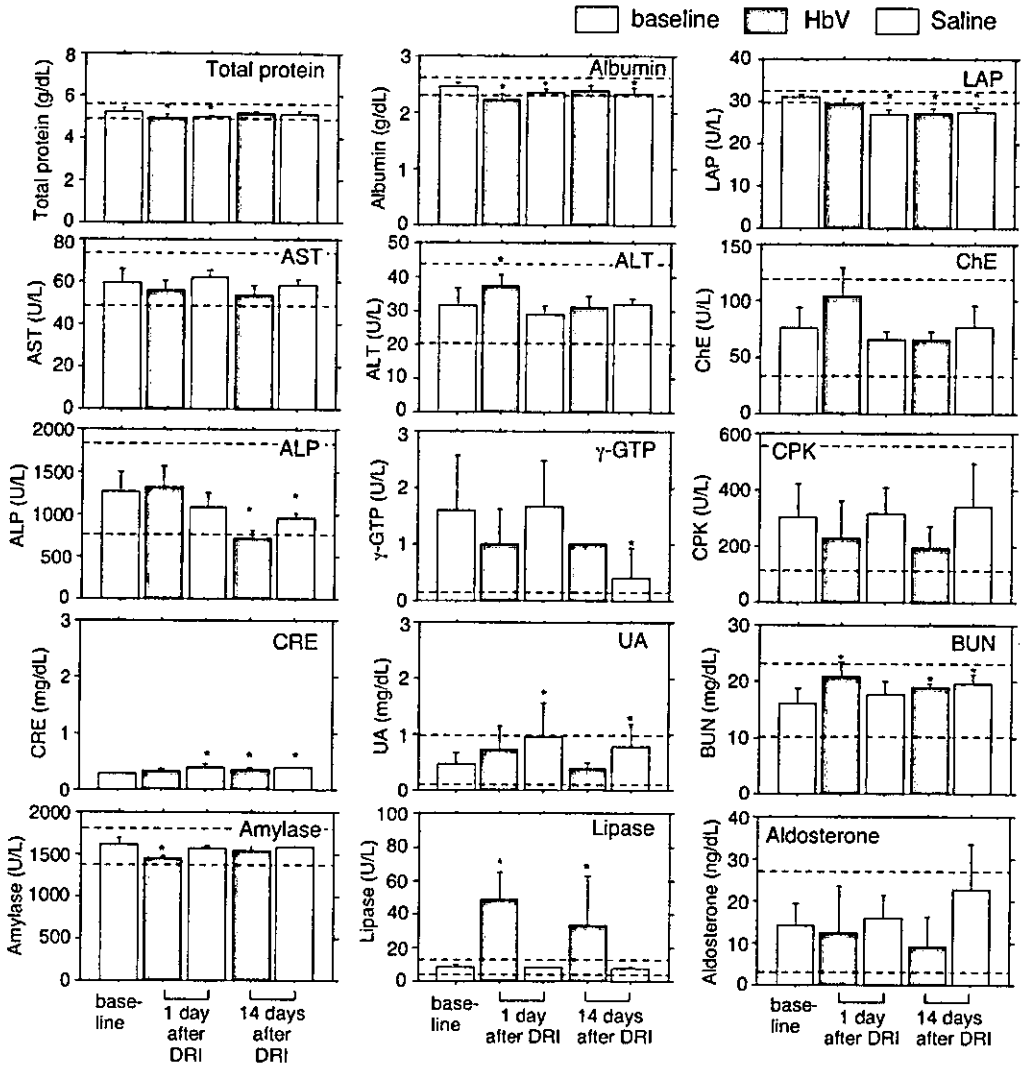

Fig. 6. Plasma clinical chemistry tests reflecting the organ functions such as liver, pancreas, and kidneys and the metabolism of Hb after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$). The dotted lines indicate the levels of 2× S.D. 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-

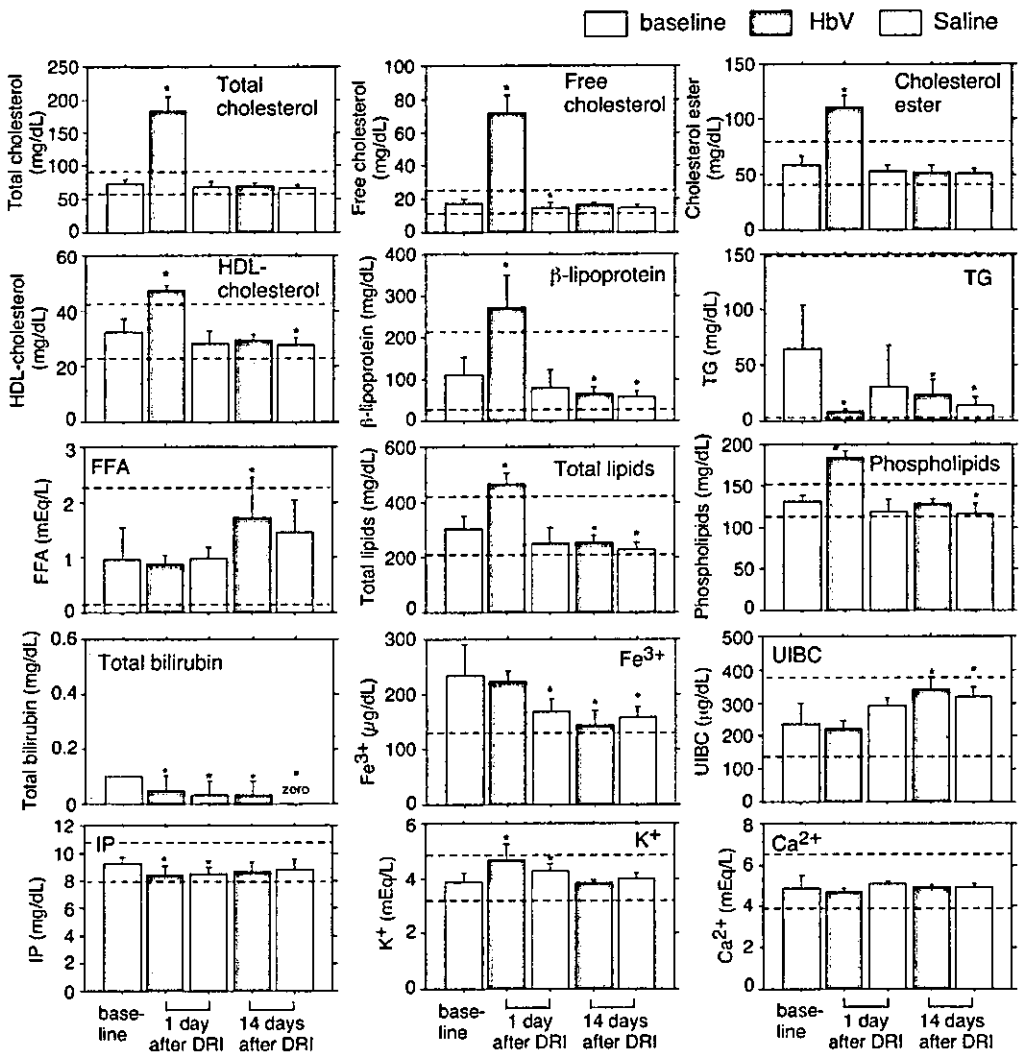

Fig. 7. Plasma clinical chemistry tests reflecting the metabolism of lipids and Hb and electrolytes 1 or 14 days after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group. The dotted lines indicate the levels of 2× S.D. TG, triglyceride; FFA, free fatty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

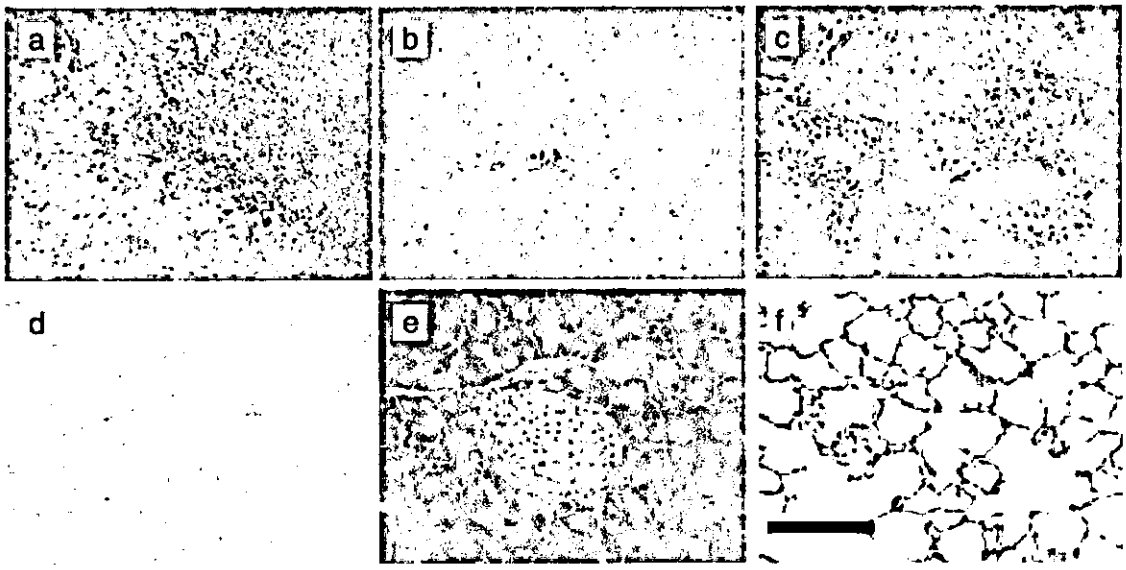

Fig. 8. Histology of spleen (a), liver (b), kidneys (c), heart (d), pancreas (e), and lungs (f) 1 day after DRI of HbV. A significant amount of HbV was accumulated in the red pulp zone of the spleen. The invasion of a significant number of Kupffer cells with HbV was seen in the liver. In the kidneys, 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).

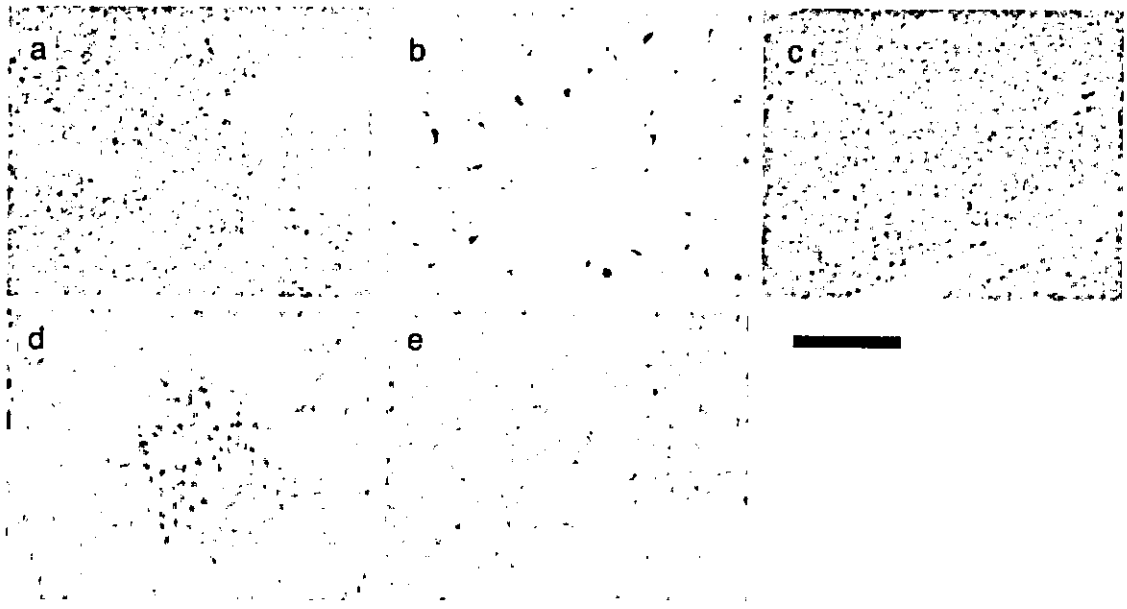

Fig. 9. Histology of spleen (a), liver (b), bone marrow (c), kidneys (d), and adrenal gland (e) 14 days after DRI. Berlin blue staining was performed to examine the presence of hemosiderin. Scale bar, 100 μm.

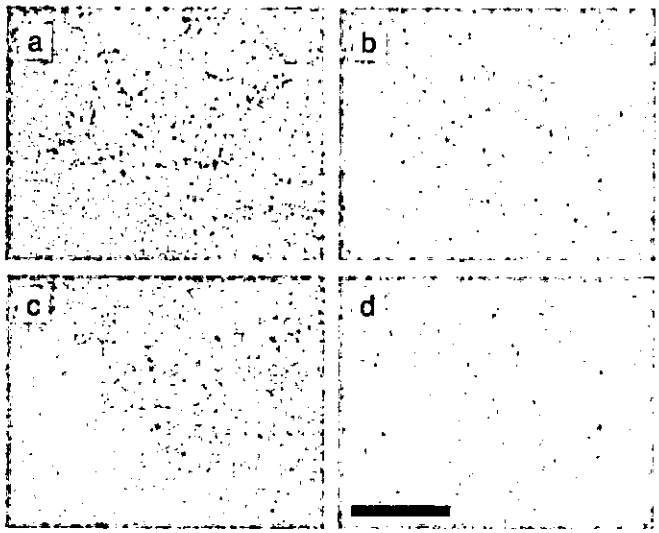

Fig. 10. Double immunohistochemical staining for HO-1 and human Hb in HbV in the rat spleen (a and c) and liver (b and d), 1 (a and b) and 14 days (c and d) after DRI of HbV. The tissues were stained with anti-rat HO-1 monoclonal antibody (GTS-3). The brown-colored portions (a, c, and d) indicate the 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 (Stueckllin-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.

Acknowledgments

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Circulation Kinetics and Organ Distribution of Hb-Vesicles Developed as a Red Blood Cell Substitute

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ABSTRACT

Phospholipid vesicles encapsulating concentrated human hemoglobin (Hb-vesicles, HbV), also known as liposomes, have a membrane structure similar to that of red blood cells (RBCs). These vesicles circulate in the bloodstream as an oxygen carrier, and their circulatory half-life times ($t_{1/2}$) and biodistribution are fundamental characteristics required for representation of their efficacy and safety as a RBC substitute. Herein, we report the pharmacokinetics of HbV and empty vesicles (EV) that do not contain Hb, in rats and rabbits to evaluate the potential of HbV as a RBC substitute. The samples were labeled with technetium-99m and then intravenously infused into animals at 14 ml/kg to measure the kinetics of HbV elimination from blood and distribution to the organs. The $t_{1/2}$ values were 34.8 and 62.6 h for HbV and 29.3 and 57.3 h for EV in rats and rabbits, respectively. At 48 h after infusion, the liver, bone marrow, and spleen of both rats and rabbits had significant concentrations of HbV and EV, and the percentages of the infused dose in these three organs were closely correlated to the circulatory half-life times in elimination phase ($t_{1/2\beta}$). Furthermore, the milligrams of HbV per gram of tissue correlated well between rats and rabbits, suggesting that the balance between organ weight and body weight is a fundamental factor determining the pharmacokinetics of HbV. This factor could be used to estimate the biodistribution and the circulation time of HbV in humans, which is estimated to be equal to that in rabbit.

Hemoglobin (Hb) isolated and purified from red blood cells (RBCs) has been tested as a principal component of RBC substitutes for carrying oxygen. However, the plasma retention time of isolated Hb is particularly short (half-life of ~0.5–1.5 h) because of the dissociation of the Hb tetramer into the dimeric form, which is subsequently filtered by the kidney, and it is known that this dimeric form is nephrotoxic (Savitsky et al., 1978). The potential of phospholipid vesicles as effective carriers of proteins and other bioactive materials has previously been proposed, since the cellular structure of such vesicles can protect the entrapped material from degradation and improve the biodistribution of proteins and other bioactive materials (Gregoriadis and Neerunjun, 1974; Papahadjopoulos et al., 1991). Phospholipid vesicles encapsulating concentrated Hb (HbV) have been proposed as a promising candidate RBC substitute, because encapsulation of Hb within a lipid membrane decreases potential side effects and toxicity of Hb, thereby making vesicles more RBC-like (Djordjevich and Miller, 1980; Gaber and Farmer, 1984; Tsuchida, 1998). The study of the safety and efficacy of HbV formulations by our research group has led to the development of an HbV formulation as a promising candidate for introduction into clinical trials (Tsuchida, 1998; Sakai et al., 2000b, 2001, 2004b; Takeoka et al., 2002).

Determination of the circulation time (half-life) of vesicles has been an important research focus, especially in RBC substitute development, because prolonged oxygen delivery is a required property for an artificial oxygen carrier. There are many reports describing the pharmacokinetics of vesicles, especially in mice and rats; however, it is difficult to apply these published data to the quantitative simulation of a clinical application. This is because of the lack of understanding of the species dependence of relevant mechanisms and correlative factors related to the clearance kinetics of vesicles. Some reports suggest that the circulatory half-life of vesicles injected in small doses into small animals such as

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ABBREVIATIONS: RBC, red blood cell; HbV, hemoglobin vesicle(s); EV, empty vesicle(s); ^{99m}Tc, technetium-99m; PEG, polyethylene glycol; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPEA, 1,5-dihexadecyl-L-glutamate-*N*-succinic acid; PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)]; PLP, pyridoxal-5′ phosphate; HMPAO, hexamethylpropyleneamine oxime; %ID, percentage of infused dose; MPS, mononuclear phagocyte system.

mice or rats empirically corresponds to half-lives that are 2 or 3 times longer in humans (Gabizon et al., 2003). In addition, the infusion dose of HbV as a RBC substitute, in terms of lipid content, is nearly a hundred times larger compared with other therapeutic uses of vesicles, even though HbV encapsulate a highly concentrated form of Hb (35–40 g/dl). Furthermore, there are many other factors such as the lipid formulation (Allen et al., 1989), vesicle size (Awasthi et al., 2003), and surface modification (Klibanov et al., 1990) that influence the circulation time and distribution of the infused vesicles. There are no clinical data available for using large infusion doses of vesicles such as those required for a RBC substitute. Therefore, we focused this research on determining the correlation factors between data from different species to simulate the pharmacokinetics of HbV. In addition, empty vesicles (EV) that do not contain Hb were studied as a reference to clarify the specific influence of encapsulated Hb on the circulation properties of the vesicles.

Scintigraphic imaging is a particularly powerful tool that can be used to develop and evaluate the formulation of vesicles (Goins and Phillips 2001). Using imaging, Phillips et al. have reported on the pharmacokinetics of liposome-encapsulated Hb radiolabeled with technetium-99m (^{99m}Tc) (Rudolph et al., 1991; Phillips et al., 1992, 1999) and achieved a formulation with long circulation times. These liposomes had a small size (<200 nm), neutral surface, and PEG modification (10 mol%), and were regarded as long-circulating vesicles (so-called stealth liposomes) ($t_{1/2}$ was 65 h after 25% intravenous top-load in rabbits) (Phillips et al., 1999). However, this particular liposome formulation had a low efficiency of Hb encapsulation, because the requisites for stealth liposomes, such as small size, neutral surface, and dense PEG modification were a disadvantage for efficient Hb encapsulation (Perkins et al., 1993; Nicholas et al., 2000). As mentioned above, the infused dose of RBC substitutes will be extremely high, so high encapsulation efficiency of Hb is essential for a successful oxygen-carrying RBC substitute. We have developed HbV with a lipid formulation and encapsulation conditions that have improved the encapsulation efficiency (Takeoka et al., 1996; Sou et al., 2003), and the present HbV formulation has an oxygen-carrying capacity equal to RBCs because of this higher encapsulation efficiency (1.7–2.0 g of Hb per gram of lipids). This article is the first report on the detailed pharmacokinetics of this HbV formulation using scintigraphic imaging of ^{99m}Tc-HbV for monitoring the circulation properties and biodistribution. Factors that would permit estimation of human pharmacokinetics of large quantities of vesicles are discussed.

Materials and Methods

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,5-dihexadecyl-L-glutamate-*N*-succinic acid (DPEA) were purchased from Nippon Fine Chemical Co., Ltd. (Osaka, Japan); 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). DPPC, cholesterol, DPEA, and PEG-DSPE were dissolved in alcohol at a molar ratio of 5, 5, 1, and 0.033, respectively, atomized, and evaporated using a spray dryer (Cracks) to prepare a lipid powder, at Nippon Fine Chemical Co., Ltd. The mixed lipid powder was hydrated with NaOH solution, submitted to three cycles of freeze-thawing, and the resultant dispersion was then lyophilized at Kanto Chemical Co. (Tokyo, Japan). The Hb solution was obtained from outdated donated blood (Japanese Red Cross) according to the purification method described previously (Sakai et al., 2002). The Hb solution (oxyhemoglobin) was converted to carbonylHb by purging the solution with 100% carbon monoxide until testing proved conversion (99% < HbCO). The final concentration of Hb was adjusted to 40 g/dl. Homocysteine, pyridoxal-5′ phosphate (PLP), and glutathione were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of HbV. HbV were prepared according to a method described previously (Takeoka et al., 1996; Tsuchida, 1998; Sakai et al., 2001; Sou et al., 2003). All HbV preparation work was performed under sterile conditions. The purified carbonylHb solution (40 g/dl) containing 5 mM homocysteine and pyridoxal-5′ phosphate [PLP/Hb ratio of 2.5 (mol/mol)] was mixed with the lyophilized powder containing the mixed lipids (DPPC, cholesterol, DPEA, and PEG-DSPE). After controlling the size of the HbV with an extrusion method (final pore size of the filter, 0.22 μm, Fuji microfilter; Fuji Photo Film Co., Tokyo, Japan), the unencapsulated Hb was removed by three ultracentrifugation steps ($10^5 g$, 30 min each). CarbonylHb was converted to OxyHb by exposure to visible light in an atmosphere of O_2. HbV were suspended in a physiological salt solution and filtered through sterilized filters (pore size, 0.45-μm Dismic; Toyo Roshi, Tokyo, Japan) and deoxygenated by bubbling with N_2 before storage (Sakai et al., 2000a). The control EV encapsulating glutathione (30 mM) was prepared using the same extrusion method.

Characterization of HbV and EV. The characteristics of HbV and EV are summarized in Table 1. The concentrations of Hb and phospholipid were determined by a cyanomethemoglobin method (Hemoglobin Test Wako; Wako Pure Chemicals, Tokyo, Japan) and the cholineoxidase method (Phospholipid C Test Wako; Wako Pure Chemicals), respectively. The encapsulation efficiency of Hb was represented as a w/w ratio of [Hb]/[lipid]. Methemoglobin and carbonylHb content were determined by spectrophotometry (Van Assendelft, 1970). The diameters of the resulting HbV (247 ± 44 nm) and EV (259 ± 32 nm) were determined using a submicron particle analyzer (N4SD; Beckman Coulter, Fullerton, CA). Endotoxin contamination was determined to be below 0.2 EU/ml by the *Limulus* assay test (Sakai et al., 2004a).

^{99m}Tc-Labeling of HbV and EV. Radiolabeling of HbV was performed according to a method described previously (Phillips et al., 1992). A saline solution of sodium [^{99m}Tc]pertechnetate (5 ml, 75 mCi) (Nycomed Amersham, San Antonio, TX) was injected into a vial containing lyophilized hexamethylpropyleneamine oxime (HMPAO, 0.5 mg, $SnCl_2$, 7.6 μg) (Ceretec; Amersham Biosciences Inc., Piscataway, NJ). The mixed solution was incubated for 5 min at room temperature. The ^{99m}Tc-HMPAO solution (1 ml) was then added to the HbV suspension ([Hb]; 10 g/dl, 1 ml), and the resulting mixed solution was incubated for 1 h. After removing free ^{99m}Tc-HMPAO by gel filtration (Sephadex-G25 column), total radioactivity was measured in a dose calibrator (Mark 5 model; Radex, Houston, TX) and the labeling efficiency (E) was calculated as the percentage of post-radioactivity in ^{99m}Tc-HbV to preradioactivity. The ^{99m}Tc-HbV suspension was mixed with unlabeled HbV suspension and the resultant HbV suspension ([Hb], 9.5 g/dl; [lipid], 4.75 g/dl) was used for the experiment. The ^{99m}Tc-EV were also prepared with same method and the lipid concentration was adjusted to the same lipid concentration as that of HbV suspension tested ([lipid], 4.75 g/dl). The ^{99m}Tc-labeled HbV and EV dispersion (0.5 ml) was mixed with rat

TABLE 1
Characteristics of ^{99m}Tc-HbV and ^{99m}Tc-EV suspensions

Parameter	^{99m}Tc-HbV	^{99m}Tc-EV
[Hb][a] (g/dl)	9.5	0
[Lipids] (g/dl)	4.75	4.75
Particle diameter (nm)	247 ± 44	259 ± 32
Endotoxin level (EU/ml)	< 0.2	< 0.2

[a] Methemoglobin, <1%; carbonylHb, <2%.

plasma (1.5 ml) from a donor rat and incubated at 37°C to check the labeling stability. A 100-μl aliquot of incubated sample at 48 h after mixing was passed through a Bio Gel A-15m (200–400 mesh) spin column. The sample was eluted by sequential addition of 100 μl of Dulbecco's phosphate-buffered saline (pH 7.3) under the centrifugal force of 1000 rpm for 1 min. Each fraction was collected separately and counted in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries, Meriden, CT). Another 100-μl aliquot of incubation sample was used as a standard. The sum total of activity eluted with HbV or EV fractions was compared with total radioactivity in the standard.

Animal Experiments. Animal experiments were performed under the National Institutes of Health Animal Use and Care guidelines and approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care Committee. Male Sprague-Dawley rats (200–274 g) were anesthetized with 3% isoflurane (VedCo, St. Joseph, MO) in 100% oxygen gas. Rats were then placed in the supine position under a Picker (Cleveland, OH) large-field-of-view gamma camera using a low-energy, all-purpose collimator and interfaced with a Pinnacle imaging computer (Medasys, Ann Arbor, MI). Image acquisition was begun as HbV or EV were infused into the tail vein at 1 ml/min. Each rat received a total dose of 0.17 to 0.37 mCi of ^{99m}Tc activity, Hb: 1.33 g/kg b.wt.; lipids: 0.67 g/kg b.wt. as an equivalent of 14 ml/kg for the HbV group (n = 5) and 0.48 to 0.55 mCi of ^{99m}Tc activity, lipids: 0.67 g/kg as 14 ml/kg for the EV group (n = 5). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.6% of body weight (Frank, 1976). The rabbit experiment was performed in the same manner. Male New Zealand White rabbits (2.2–2.9 kg) were anesthetized with an intramuscular injection of ketamine/xylazine (both from Phoenix Scientific, St. Joseph, MO) mixture (50 and 10 mg/kg body weight, respectively). One ear of a rabbit was catheterized with a venous line, and the other ear was catheterized with an arterial line. HbV or EV was infused in the venous line at 1 ml/min under the same gamma camera, and the blood samples were drawn from the arterial line. Each rabbit received a total dose of 3.7 to 4.5 mCi of ^{99m}Tc activity, Hb: 1.36 g/kg b.wt.; lipids: 0.68 g/kg b.wt. as 14.25 ml/kg for the HbV group (n = 5) and 3.5 to 4.9 mCi, lipids: 0.68 g/kg as 14.25 ml/kg for the EV group (n = 4). The infused dose (in volume) was estimated to be 25% of blood volume where the total blood volume was assumed to be 5.7% of body weight (Kozma et al., 1974).

Image Analysis. One-minute dynamic 64 × 64 pixel scintigraphic images were acquired over a continuous period of 0.5 and 2 h for rats and rabbits after the infusion of HbV or EV, respectively. Static images were also acquired at 3, 6, 12, 24, 36, and 48 h postinfusion. The image analysis was performed using a nuclear medicine analysis workstation (Pinnacle computer; Medasys). The regions of interest were drawn over the whole body, liver, and spleen in images. The counts of radioactivity were decay-corrected at each time and converted to a percentage of the whole body counts. Corrections were made for the blood pool contribution of the liver and spleen of the rat (17 and 6%, respectively, of the total blood volume). For rabbit, the liver was corrected by 25.4% of the total blood volume, and the spleen was individually corrected by 1.047 ± 0.076% for HbV and 1.592 ± 0.049% of the total blood volume for EV as percentage of infused dose (%ID) just after infusion, respectively.

Blood Persistence and Biodistribution. Blood was collected from the tail vein of the rat or arterial line of the rabbit (50 or 100 μl) at various times postinfusion. The radioactivity of blood samples was quantified in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries) at the same time. The counts at each time were converted to the percentage of the counts of sample collected immediately after infusion. The elimination rate constants (k) were calculated by the least-squares method and half-life time ($t_{1/2}$) was calculated from eq. 1.

$$t_{1/2} = \frac{0.693}{k} \quad (1)$$

The animals were rapidly sacrificed at 48 h, and the tissue samples were collected, weighed, and counted for radioactivity in a scintillation well counter (Canberra multichannel analyzer; Canberra Industries) to calculate the biodistribution. To calculate the %ID per organ, total blood volume, muscle, and skin mass were estimated as 5.6, 40, and 13% of total body weight for rat (Frank, 1976; Petty, 1982), and 5.7, 45, and 10% of total body weight for rabbit (Kozma et al., 1974; Kaplan and Timmons, 1979), respectively. The bone was estimated as 10% of total body weight for rat (Frank, 1976; Petty, 1982) and 12 times the femur weight for rabbit (Dietz, 1944).

Estimation of the Biodistribution in Humans. The total Hb or lipids per organ (Ws) was calculated from the %ID and ID of Hb or lipids in terms of weight.

$$Ws(\text{mg}) = \frac{\%\text{ID} \times \text{ID}}{100} \quad (2)$$

The organ weight (Wo) of experimental animals was measured by an electronic balance and the Hb per organ weight (R) was calculated.

$$R(\text{mg/g}) = \frac{Ws}{Wo} \quad (3)$$

Ws was calculated from eq. 3 for humans, where the weights of liver, spleen, and bone (Wo) were estimated as 1.8, 0.18, and 5.0 kg, respectively, for average humans (70 kg) (International Commission on Radiological Protection, 1984), and the R value was applied as an average value between rats and rabbits shown in Table 4 for each organ. The ID of HbV ([Hb] = 9.5 g/dl, [lipids] = 4.75 g/dl) was calculated to be 25% of the blood volume (4.9 liters, 70 ml/kg b.wt.), and the %ID was calculated from eq. 2. The half-life times ($t_{1/2\beta}$) were estimated from eq. 4, where, constant value (C) was determined as a slope of the fitting line in this study and $\%ID_{total}$ was sum values of %ID for liver, spleen, and bone.

$$t_{1/2\beta} = \frac{C}{\%\text{ID}_{\text{total}}} \quad (4)$$

Statistical Methods. Values are reported as mean ± S.E.M. Statistical analysis was performed using Microsoft Excel for Windows. The image analysis and biodistribution data were compared using the Student's unpaired t test. A P value <0.01 or 0.05 was considered statistically significant.

Results

Labeling Efficiencies. The labeling efficiencies of ^{99m}Tc-HbV and ^{99m}Tc-EV were 69.1 ± 2.0% (n = 2) and 75.6 ± 5.1% (n = 3) for the rat studies, and 62.0 ± 4.8% (n = 5) and 70.9 ± 2.1% (n = 2) for the rabbit studies. Labeling efficiencies were similar for both ^{99m}Tc-HbV and ^{99m}Tc-EV, even though ^{99m}Tc-HbV used homocysteine and ^{99m}Tc-EV used glutathione. The ^{99m}Tc would be located in the inner aqueous phase of vesicles, and both homocysteine and Hb of HbV, and glutathione of EV would possibly bind the ^{99m}Tc (Rudolph et al., 1991; Phillips et al., 1992). The incubation of labeled HbV and EV in serum for 48 h revealed that 5 and 4% of the ^{99m}Tc dissociated from HbV and EV, indicating that the labeling was very stable and the contents were stably encapsulated inside the vesicles.

Circulation Kinetics. To determine the circulation kinetics as shown in Fig. 1, a and b, the radioactive counts of blood samples were plotted as a percentage of the counts for blood sample collected immediately at the end of the infusion with

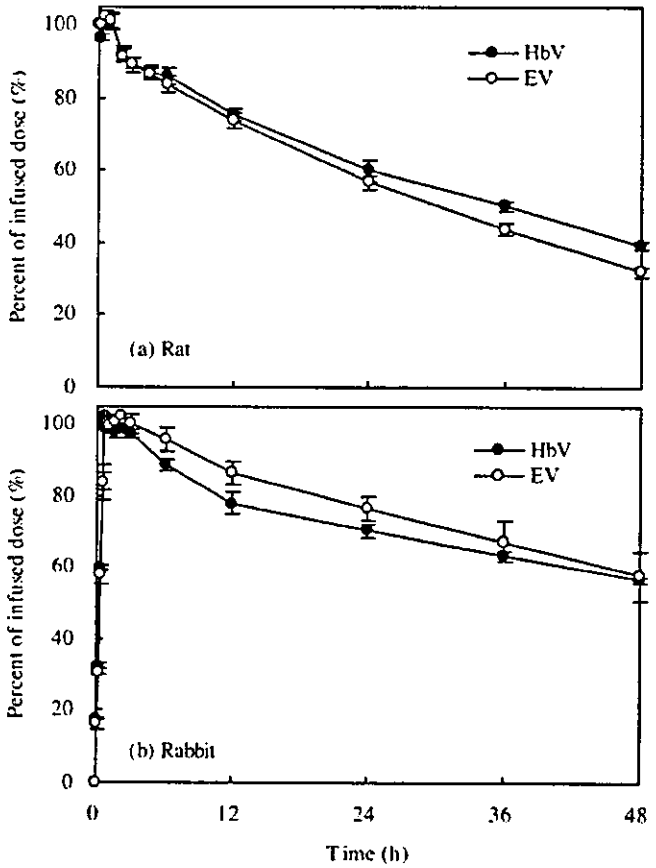

Fig. 1. Circulation kinetics of HbV and EV after top-loading intravenous infusion (14 ml/kg) in rats and rabbits. The radioactivity was determined by scintillation counting of blood samples with time. The percentage of radioactivity is calculated as a percentage of baseline radioactivity in a blood sample withdrawn just after HbV or EV infusion.

time. The elimination profiles of infused HbV showed two components with an initial fast clearance followed by a slower clearance phase, which is regarded as a distribution (α) phase in the mononuclear phagocyte system (MPS) and an elimination (β) phase, respectively. The clearance rate constant in the distribution phase of HbV was equal to that of EV, and k_β was 1.3 times smaller than that of EV in rats as shown in Table 2. The circulation half-life times ($t_{1/2}$ values) associated with both the distribution and elimination phases of HbV and EV in rats were 34.8 and 29.3 h, respectively. The clearance rates of HbV and EV were slower in rabbits compared with those in rats, especially for the distribution phase. The k_α of HbV was 0.0226 h^{-1} in rabbit, which was one-quarter of that in rats and 1.4 times larger than that of EV in rabbit. k_β for HbV was 1.3 times smaller than that of EV. The $t_{1/2}$ values of HbV and EV were 62.6 and 57.3 h in rabbits, respectively.

Imaging Study. The gamma camera images of rats or rabbits receiving HbV were acquired at various times to determine the organ distribution profiles with time. As shown in Figs. 2 and 3, radioactivity was observed over the whole body of animals and in the heart, demonstrating that HbV were circulating. Immediately after infusion, the heart, liver, and spleen were identified because these organs had a large blood pool volume, and the relative intensities of the liver and spleen increased in comparison with the heart with time. The %ID in liver and spleen calculated from gamma camera images with decay correction and correction for blood pool contribution are shown in Fig. 4. The %ID in liver was increased during the infusion and decreased after the infusion ended, especially in HbV as shown in Fig. 4, a and c. This initial decrease was most likely due to the adjustment of blood volume after top-loading. The values of %ID in liver and spleen were quickly increased during the first 6 to 12 h after infusion and reached a plateau at 48 h. At 48 h, the liver had 10.9 ± 0.8 and 7.6 ± 1.0% of HbV in rats and rabbits, respectively, whereas the spleen had 6.6 ± 0.3 and 0.98 ± 0.14% of HbV in rats and rabbits, respectively.

Biodistribution. The detailed biodistribution data of HbV at 48 h are shown in Table 3. HbV could be precipitated easily by ultracentrifugation of blood sample, and no Hb was detected in the supernatant serum in the blood sample for 48 h. In addition, no Hb was detected in urine for 48 h supporting that the Hb was not eluted from vesicles during circulation. HbV and EV were mainly distributed in liver, bone marrow, and spleen, and the %ID values for HbV were smaller than those of EV in these organs. Relatively high values for the bowel, feces, and urine were likely due to metabolism during excretion of HbV. The sum values of %ID for liver, spleen, and bone ($\%ID_{total}$), which are the main organs for MPS uptake, were 26.60 and 13.64% for HbV and 36.36 and 17.84% for EV in rats and rabbit, respectively. The corresponding $t_{1/2\beta}$ values given in Table 2 were 39.1, 79.2, 30.1, and 60.2 h, respectively. These $t_{1/2\beta}$ values are in proportion to the reciprocal of $\%ID_{total}$ as shown in Fig. 5, and the constant value (C) in eq. 4 was determined to be 1074.1 as a slope of the fitting line.

The calculated total lipids and Hb doses (in milligrams) delivered to the liver, bone, and spleen are summarized in Table 4. These values are independent of the species dependence of relative weight balances of organs in whole body and represent the amount of uptake of the HbV in a gram of each organ. The spleen had 14.43 ± 0.54 and 14.92 ± 1.25 mg of Hb per gram in rat and rabbit, and the liver and bone also had similar values in rat and rabbit.

Discussion

The improvement in oxygen-carrying capacity of HbV as a RBC substitute requires longer circulation and a higher en-

TABLE 2
Kinetic parameters of HbV and EV clearance from blood in rats and rabbits (25% top-loading)

Animal	Sample	Distribution (α) Phase		Elimination (β) Phase		$t_{1/2}$
		k_α	$t_{1/2\alpha}$	k_β	$t_{1/2\beta}$	
		h^{-1}	h	h^{-1}	h	
Rat	HbV	0.0894	7.8	0.0177	39.1	34.8
	EV	0.1004	6.9	0.0230	30.1	29.3
Rabbit	HbV	0.0226	30.7	0.0088	79.2	62.6
	EV	0.0159	43.6	0.0115	60.2	57.3

TABLE 3
Biodistribution of HbV and EV as a percentage of the infused dose per organ (%ID/organ) and percentage of the infused dose per gram of organ (%ID/g organ) at 48 h after 25% top-loading in rats or rabbits

Organ	Rat		Rabbit	
	HbV	EV	HbV	EV
%ID/organ ± S.E.M.				
Blood	33.27 ± 1.11*	24.13 ± 0.65	50.95 ± 2.02†	52.76 ± 4.80‡
Liver	10.04 ± 0.86*	14.13 ± 0.40	7.55 ± 0.46†	8.64 ± 0.34‡
Bone	10.06 ± 0.21*	13.05 ± 0.38	5.37 ± 0.33*†	7.36 ± 0.28‡
Spleen	6.50 ± 0.30*	9.18 ± 0.37	0.72 ± 0.10*†	1.84 ± 0.28‡
Bowels	7.30 ± 1.59	4.16 ± 0.35	9.61 ± 2.31	8.62 ± 4.42
Skin	2.37 ± 0.33	2.29 ± 0.12	0.88 ± 0.05†	1.09 ± 0.21‡
Kidney	2.40 ± 0.10*	3.35 ± 0.08	1.47 ± 0.13†	1.69 ± 0.21‡
Muscle	1.94 ± 0.28	1.98 ± 0.27	2.51 ± 0.31	2.62 ± 0.76
Lung	0.62 ± 0.03	0.54 ± 0.03	0.55 ± 0.02	0.43 ± 0.06
Heart	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.01†	0.13 ± 0.02
Brain	0.16 ± 0.01*	0.09 ± 0.01	0.08 ± 0.01*†	0.05 ± 0.00‡
Testis	0.12 ± 0.01*	0.09 ± 0.01	0.06 ± 0.02†	0.07 ± 0.01
Feces	9.50 ± 1.17	6.95 ± 0.29	5.06 ± 2.56	2.02 ± 0.55‡
Urine	13.61 ± 0.31	12.87 ± 0.41	11.30 ± 1.22	7.81 ± 1.44‡
%ID/g organ ± S.E.M.				
Blood	2.919 ± 0.032	1.706 ± 0.044	0.356 ± 0.017	0.354 ± 0.030
Liver	1.244 ± 0.096	1.378 ± 0.045	0.093 ± 0.004	0.131 ± 0.019
Bone	0.497 ± 0.021	0.518 ± 0.020	0.043 ± 0.003	0.062 ± 0.002
Spleen	10.059 ± 0.072	10.790 ± 0.402	0.823 ± 0.072	1.483 ± 0.072
Bowels	0.390 ± 0.073	0.202 ± 0.008	0.031 ± 0.006	0.029 ± 0.014
Skin	0.091 ± 0.014	0.070 ± 0.004	0.004 ± 0.000	0.004 ± 0.001
Kidney	1.604 ± 0.057	1.839 ± 0.055	0.089 ± 0.005	0.110 ± 0.017
Muscle	0.024 ± 0.003	0.020 ± 0.003	0.002 ± 0.000	0.002 ± 0.001
Lung	0.619 ± 0.022	0.458 ± 0.014	0.068 ± 0.004	0.057 ± 0.012
Heart	0.264 ± 0.009	0.187 ± 0.012	0.026 ± 0.002	0.027 ± 0.006
Brain	0.111 ± 0.010	0.062 ± 0.003	0.011 ± 0.001	0.006 ± 0.001
Testis	0.042 ± 0.002	0.027 ± 0.001	0.013 ± 0.002	0.016 ± 0.003

* Difference is statistically significant from EV in same species at $P < 0.01$.
† Difference is statistically significant from HbV in rat at $P < 0.05$.
‡ Difference is statistically significant from EV in rat at $P < 0.05$.

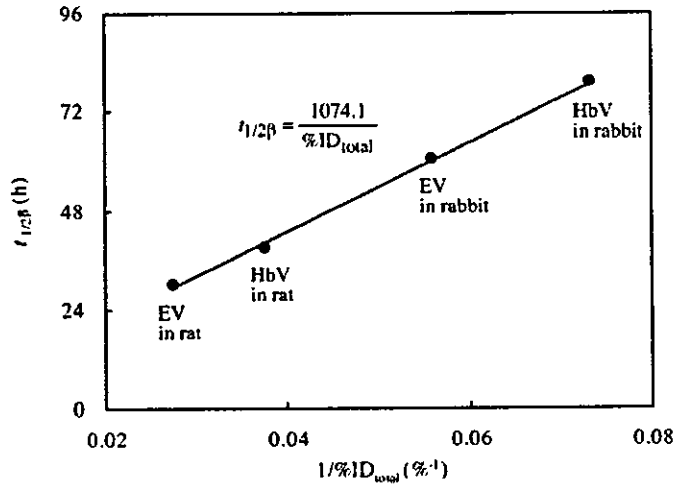

Fig. 5. Proportional relationship between the circulation half-life time ($t_{1/2\beta}$) and the reciprocal of $\%ID_{total}$ in the elimination phase. The $\%ID_{total}$ was calculated as a sum value of %ID in liver, bone, and spleen at 48 h. The fitting line was determined by the regression analysis (coefficient of determination; $R^2 = 0.9985$).

([Hb], 9.5 g/dl; [lipid], 4.75 g/dl). The normal range of human organ weight is relatively wide such as 1.4 to 1.8 kg (liver) and 0.08 to 0.3 kg (spleen), so the $t_{1/2\beta}$ would be varied around 3 days. This $t_{1/2\beta}$ is approximately two times larger than that of rat, and this ratio almost follows that derived from empirical speculation (Gabizon et al., 2003). This method of estimating vesicle circulation kinetics and organ uptake in different animal species may be useful for all types of vesicle (liposome) formulations that are currently under development as drug delivery vehicles. More studies will be required to further validate this method of estimating circulation kinetics and organ uptake in different animal species.

The development of RBC substitutes is progressing, and some modified Hbs have been studied in clinical trials. The reported $t_{1/2}$ value was 23 h for polymerized bovine Hb (Hughes et al., 1995), 16 to 20 h for *o*-raffinose-cross-linked and polymerized human Hb (Carmichael et al., 2000), and 24 h for glutaraldehyde-cross-linked and polymerized human Hb (Gould et al., 1998). Even though HbV have not yet been tested clinically, we have demonstrated in the present report that HbV have significantly improved properties, based on their circulation kinetics and biodistribution, suggesting their improved safety and efficacy as a RBC substitute. In addition, the successful application of vesicles as RBC substitutes at this large infusion dose suggests a promising future for vesicles (liposomes), and the present formulation would potentially be available not only as a RBC substitute but also for various applications such as drug delivery systems.

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TABLE 4

Comparison of HbV and EV as milligrams of lipids per gram of organ and milligrams of Hb per gram of organ at 48 h after 25% top-loading in rats or rabbits

Organ	HbV in Rat		HbV in Rabbit		EV in Rat	EV in Rabbit
	mg lipids/g organ[a]	*mg Hb/g organ*[b]	*mg lipids/g organ*[a]	*mg Hb/g organ*[b]	*mg lipids/g organ*[a]	
Blood	4.23 ± 0.20	8.40 ± 0.40	6.47 ± 0.24†	12.93 ± 0.48†	2.94 ± 0.06	6.55 ± 0.64†
Liver	1.79 ± 0.12	3.56 ± 0.23	1.68 ± 0.06	3.36 ± 0.12	2.38 ± 0.06	2.24 ± 0.18
Bone	0.72 ± 0.01	1.42 ± 0.02	0.78 ± 0.05	1.57 ± 0.09	0.89 ± 0.04	1.09 ± 0.08
Spleen	14.43 ± 0.54	28.63 ± 1.06	14.92 ± 1.25	29.85 ± 2.50	18.58 ± 0.51	25.83 ± 1.43†

† Difference is statistically significant from HbV in rat at $P < 0.05$.
[a] Calculated values from ID of lipids and %ID/g organ.
[b] Calculated values from ID of Hb and %ID/g organ.

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Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution

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Cabrales, Pedro, Hiromi Sakai, Amy G. Tsai, Shinji Takeoka, Eishun Tsuchida, and Marcos Intaglietta. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am J Physiol Heart Circ Physiol* 288: H1885–H1892, 2005. First published November 24, 2004; doi:10.1152/ajpheart.01004.2004.—The oxygen transport capacity of phospholipid vesicles encapsulating purified Hb (HbV) produced with a P_{O_2} at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) was investigated in the hamster chamber window model by using microvascular measurements to determine oxygen delivery during extreme hemodilution. Two isovolemic hemodilution steps were performed with 5% recombinant albumin (rHSA) until Hct was 35% of baseline. Isovolemic exchange was continued using HbV suspended in rHSA solution to a total [Hb] of 5.7 g/dl in blood. P_{50} was modified by coencapsulating pyridoxal 5′-phosphate. Final Hct was 11% for the HbV groups, with a plasma [Hb] of 2.1 ± 0.1 g/dl after exchange with HbV_8 or HbV_{29}. A reference group was hemodiluted to Hct 11% with only rHSA. All groups showed stable blood pressure and heart rate. Arterial oxygen tensions were significantly higher than baseline for the HbV groups and the rHSA group and significantly lower for the HbV groups compared with the rHSA group. Blood pressure was significantly higher for the HbV_8 group compared with the HbV_{29} group. Arteriolar and venular blood flows were significantly higher than baseline for the HbV groups. Microvascular oxygen delivery and extraction were similar for the HbV groups but lower for the rHSA group ($P < 0.05$). Venular and tissue P_{O_2} were statistically higher for the HbV_8 vs. the HbV_{29} and rHSA groups ($P < 0.05$). Improved tissue P_{O_2} is obtained when red blood cells deliver oxygen in combination with a high- rather than low-affinity oxygen carrier.

oxygen-carrying capacity; blood substitutes; tissue oxygen; hemoglobin oxygen affinity

PHOSPHOLIPID VESICLES encapsulating concentrated hemoglobin (Hb) solution [Hb vesicles (HbV) or liposome-encapsulated Hb] provide oxygen-carrying capacity to plasma expanders, reproducing several of the characteristics of red blood cells (RBC) suspended in plasma. HbV contain Hb at a high concentration within a cell membrane-like structure. Their oxygen dissociation curve can be adjusted by varying the concentration of pyridoxal 5′-phosphate (PLP). A widely accepted premise for designing a blood substitute is that its Hb should have an oxygen dissociation curve like that of RBC or one that is right shifted, i.e., having a high P_{50} to facilitate the unloading of oxygen (P_{50} is the partial pressure of oxygen at which the Hb molecule is 50% saturated). In a previous study by Sakai et al. (16), vesicles were formulated with P_{50} values set at 9, 16, and 30 mmHg. The study showed that optimal tissue oxygen conditions were obtained when 80% of the circulating blood was substituted with HbV whose P_{50} was 16 mmHg, a value considerably lower than the usual value of 28 mmHg for normal blood (16). Oxygen-carrying capacity was found to be well above the oxygen supply limitation.

Recent developments in the field of oxygen-carrying plasma expanders (OCPE) based on molecular Hb solutions reported by Tsai et al. (22) show that the addition of comparatively small amounts of a significantly left-shifted polyethylene glycol-conjugated oxygen carrier (P_{50} ~5 mmHg) to blood in extreme hemodilution leads to baseline microvascular and systemic conditions. This result could not be obtained in identical extreme hemodilution experiments with the use of a right-shifted molecular Hb solution at a considerably higher concentration (19).

Extreme hemodilution in the hamster window chamber model to a hematocrit (Hct) level of ~11% is a powerful tool to test the efficacy of OCPEs in restoring microvascular function and systemic conditions. This Hct is below the threshold at which the organism becomes oxygen supply limited (5, 22, 23). In this scenario, the effects of a blood substitute became magnified upon introduction into the circulation. Furthermore, by encapsulating Hb, a phospholipid vesicle eliminates the problem of Hb extravasation and provides a setting in which the biophysical properties of the infusion solution can be rigorously controlled while allowing for the change in P_{50}. Therefore, experimenting with vesicles that encapsulate Hb formulated with different P_{50} values provides the unique opportunity to investigate how oxygen affinity regulates oxygen delivery to the tissue by the microcirculation, a value not attainable by lowering RBC Hb P_{50} by the administration of sodium cyanate, which may introduce changes in tissue metabolism (7). In addition, RBC and HbV are different in size, flow pattern, homogeneous distribution in the plasma phase, and the mechanism of oxygen unloading in capillaries, and direct comparison between RBC and HbV is impossible. All these conditions indicate that the optimal P_{50} should be different in HbV and RBC.

In the present study, we investigated the microvascular effects of restoring oxygen-carrying capacity in conditions of extreme hemodilution, introducing by exchange transfusion identical amounts of Hb-carrying vesicles in which oxygen affinity was specifically controlled so that P_{50} was either 8 or 29 mmHg. The P_{50} value of 8 mmHg was chosen because it is

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similar to that of a recently developed oxygen carrier that is effective at a low concentration (2–4, 22). In these experiments, the hemodilution protocols were performed using a recombinant albumin solution (13) as the plasma expander.

METHODS

Investigations were performed in male golden Syrian hamsters (55–65 g body wt) fitted with a dorsal skinfold chamber window (6). This model has been used extensively for investigations of the intact microvasculature of adipose and subcutaneous tissue and skeletal muscle in conscious animals for extended periods. Pentobarbital sodium (50 mg/kg ip) was used for window implantation and for carotid artery and jugular vein catheterization. The microvasculature was examined 4–5 days after the initial surgery, and only animals passing an established systemic and microcirculatory inclusion criteria, which included having tissue void of low perfusion, inflammation, and edema (21), were entered into the study. Animal handling and care followed the NIH *Guide for the Care and Use of Laboratory Animals*. The experimental protocol was approved by the local animal care committee.

Preparation of HbV with different P_{50}. HbV were prepared under sterile conditions as previously reported (12, 15). 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 purified Hb (38 g/dl) contained 0 or 14.7 mM PLP (Sigma Chemical, St. Louis, MO) as an allosteric effector at a molar ratio of [PLP]/[Hb] = 0 or 2.5, respectively. The lipid bilayer was composed of 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, Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (0.3 mol% of the total lipid; NOF, Tokyo, Japan) (17). HbV with a 250-nm diameter were suspended in a physiological saline solution in which [Hb] = 10 g/dl, sterilized with filters (Dismic, pore size 0.45 μm; Toyo Roshi, Tokyo, Japan), and deoxygenated with N_2 bubbling for storage (14). The content of lipopolysaccharide was <0.1 EU/ml.

Before use, the HbV suspension ([Hb] = 10 g/dl, 8.6 ml) was mixed with a solution of recombinant human serum albumin (rHSA 25%, 1.4 ml; Nipro, Osaka, Japan) to regulate the rHSA concentration in the suspending medium of the vesicles to 5 g/dl. Under this condition, the colloid osmotic pressure of the suspension is ~20 mmHg (Wescor 4420 colloid osmometer; Wescor, Logan, UT) (12). As a result, the Hb concentration of the suspension was 8.6 g/dl.

In a previous study (16), HbV were suspended in 8 g/dl HSA. However, we changed to 5 g/dl rHSA because it showed better microvascular perfusion in the hamster window model (i.e., increased red cell velocity and functional capillary density) than 8 g/dl HSA. The suspension was filtered through sterile filters (pore size 0.45 μm; Millipore, Billerica, MA). The characteristics of HbV are listed in Table 1, with all parameters being almost identical except oxygen affinity (HbV_8, P_{50} = 8 mmHg; HbV_{29}, P_{50} = 29 mmHg).

Table 1. *Physical characteristics of solutions*

Fluid	Viscosity, cp	COP, mmHg	P_{50}, mmHg
rHSA (5%)	0.98	20	
HbV_8 (10 g Hb/dl)	2.92		8
HbV_{29} (10 g Hb/dl)	2.96		29
HbV_8/rHSA (8.6 g Hb/dl)	2.87	20	8
HbV_{29}/rHSA (8.6 g Hb/dl)	2.90	20	29

Viscosity was measured at a shear rate of 160 s^{-1} at 37°C. COP, colloid osmotic pressure measured at 27°C; P_{50}, partial pressure of oxygen at which Hb is 50% saturated; rHSA, recombinant human serum albumin; HbV_8 and HbV_{29}, Hb vesicles with a P_{50} of 8 and 29 mmHg, respectively.

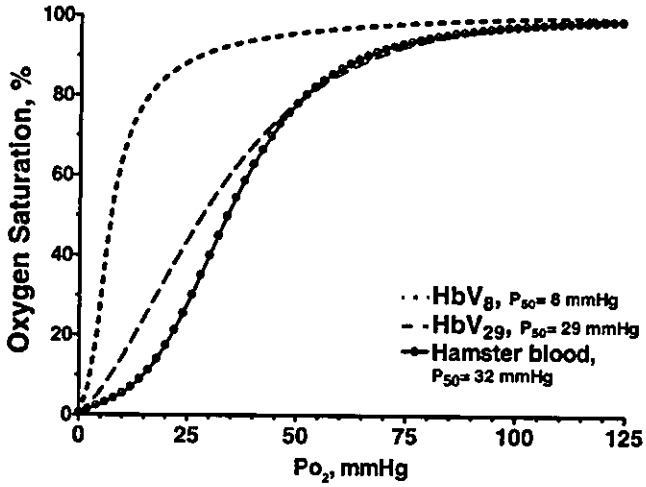

Fig. 1. Oxygen dissociation curves for phospholipid vesicles encapsulating purified Hb (HbV) produced with a Po_2 at which Hb is 50% saturated (P_{50}) of 8 (HbV_8) and 29 mmHg (HbV_{29}) vs. the dissociation curve for hamster blood (P_{50} = 32 mmHg).

Measurements of P_{50} and rate of oxygen release from HbV. The P_{50} and Hill number of each HbV and Hb solution were calculated from oxygen dissociation curves measured with a Hemox analyzer (TCS-Medical Products) at 37°C (Fig. 1).

Acute isovolemic exchange-transfusion (hemodilution) protocol. Progressive hemodilution to a final systemic Hct level of 11% was accomplished with three isovolemic exchange steps. This protocol, leading to extreme hemodilution while maintaining stable hemodynamic conditions, is described in detail in a previous report by Tsai (19). Briefly, the volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of the body weight. An acute anemic state was induced by lowering systemic Hct by 60% with two steps of progressive isovolemic hemodilution using 5% rHSA, referred to as exchange *levels 1* and *2*. *Level 1* exchange was 40% of blood volume, and *level 2* and *3* exchanges were 35% of blood volume, respectively.

After *level 2*, the animals were randomly divided into three experimental groups by being assigned to an experimental group according to a sorting scheme based on a list of random numbers (1). *Level 2* exchange was followed by *level 3* exchange. Hemodilution with 5% rHSA solution was continued with one group of the *level 2* hemodiluted animals, the experimental group rHSA, until Hct was decreased to 11% of baseline (Fig. 2). The test materials were studied by assigning the remainder of the *level 2* animals to groups labeled HbV_8 (P_{50} = 8 mmHg) and HbV_{29} (P_{50} = 29 mmHg) and were hemodiluted using these materials, reducing Hct to 11%. Plasma Hb concentrations derived for HbV_8 and HbV_{29} after exchange of 35% blood volume are estimated around 2.0–2.3 g/dl for both groups (35% of estimated total Hb content) (21).

Because mixed blood is withdrawn during the exchanges, a 110% blood volume exchange was needed to reduce Hct to 25% of baseline (11% Hct). Test solutions were infused into the jugular vein catheter after passing through an in-line, 13-mm-diameter, 0.2-μm syringe filter at a rate of 100 μl/min. Blood was simultaneously withdrawn using a dual syringe pump ("33" syringe pump; Harvard Apparatus, Holliston, MA) at the same (isovolemic-normovolemic) rate from the carotid artery catheter (4, 5, 19). This slow rate of exchange provided for a stable mean arterial pressure immediately after the exchange. Each animal was allowed a 10-min stabilization period before data acquisition.

Blood chemistry and biophysical properties. Arterial blood was collected in heparinized glass capillaries (0.05 ml) and immediately

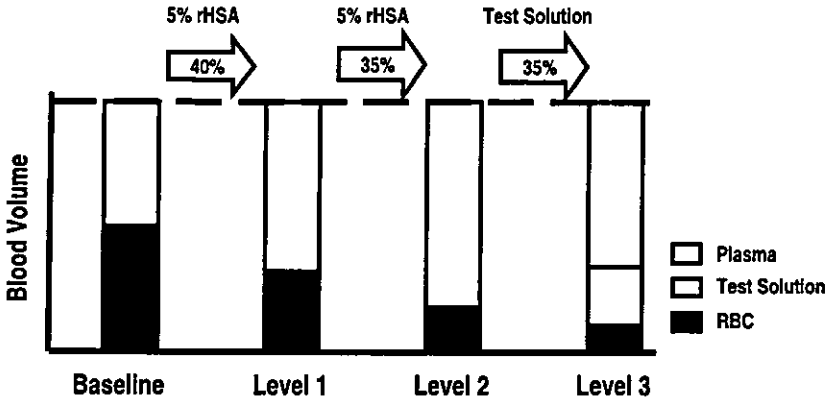

Fig. 2. Hemodilution was attained by means of a progressive, stepwise, isovolemic blood exchange-transfusion protocol. Volume of each exchange-transfusion step was calculated as a percentage of the blood volume, estimated as 7% of body weight. An acute anemic state was induced by lowering systemic Hct, using a 5% recombinant human serum albumin (rHSA) solution, in 2 progressive steps of isovolemic hemodilution labeled *level 1* and *level 2* exchanges. *Level 3* exchange was achieved by a third hemodilution that continued using rHSA or the vesicle solutions HbV_8 or HbV_{29} suspended in 5% rHSA (test solutions). RBC, red blood cells.

analyzed for arterial Po_2 (Pa_{O_2}), arterial Pco_2 (Pa_{CO_2}), base excess (BE), and pH (Blood Chemistry Analyzer 248; Bayer, Norwood, MA). The comparatively low Pa_{O_2} and high Pa_{CO_2} values of these animals is a consequence of their adaptation to a fossorial environment. Blood samples for viscosity and colloid osmotic pressure measurements were quickly withdrawn from the animal with a heparinized 5-ml syringe at the end of the experiment for immediate analysis.

Viscosity was measured in a cone/plate viscometer (DV-II+) with a cone spindle (CPE-40; both from Brookfield Engineering Laboratories, Middleboro, MA) at a shear rate of 160 s^{-1}. Colloid osmotic pressure (COP) was measured using the Wescor 4420 colloid osmometer (23).

Functional capillary density. Functional capillary density (FCD; in cm^{-1}) is the total length of RBC-perfused capillaries divided by the area of the microscopic field of view (21). Capillary segments were considered functional if RBC were observed to transit over a 30-s period. FCD was tabulated from the capillary lengths with RBC flow in an area comprising 10 successive microscopic fields (420 × 320 μm). Detailed mappings were made of the chamber vasculature to study the same microvessels throughout the experiment.

Microhemodynamic parameters. Arteriolar and venular blood flow velocities were measured online using the photodiode cross-correlation technique (8) (Fiber Optic Photo Diode and Velocity Tracker Correlator model 102B; Vista Electronics, Ramona, CA). The centerline velocity (V) was corrected according to vessel size to obtain the mean RBC velocity (11). The video image shearing technique was used to measure vessel diameter (D) online. Blood flow was calculated from the measured parameters as $(Q) = V\pi(D/2)^2$.

Microvascular Po_2 distribution. High-resolution microvascular Po_2 measurements were made using phosphorescence-quenching microscopy (18), a method based on the oxygen-dependent quenching of phosphorescence emitted by albumin-bound metalloporphyrin complex after pulsed light excitation. Phosphorescence microscopy is not dependent on the level of dye within the tissue, and the decay time is inversely proportional to the Po_2 level. The phosphorescence decay curves were converted to oxygen tensions by using a fluorescence decay curve fitter (model 802; Vista Electronics) (9). This technique has been used in this animal preparation and others for both intravascular and extravascular oxygen tension measurements, because albumin exchange between plasma and tissue allows for sufficient concentrations of albumin-bound dye within the interstitium to achieve an adequate signal-to-noise ratio. Animals received a slow intravenous injection of 15 mg/kg body wt at a concentration of 10.1 mg/ml of a palladium-*meso*-tetra(4-carboxyphenyl)porphyrin (Porphyrin Products, Logan, UT). Po_2 measurements were made 20 min after porphyrin injection, allowing it to be distributed to all the tissues.

In our system, intravascular measurements are made by placing an optical rectangular window (5 × 40 μm) within the vessel of interest, with the longest side of the rectangular slit positioned parallel to the vessel wall. Tissue Po_2 is measured in regions void of large vessels within intercapillary spaces with an optical window size of ~10 × 10 μm, which allows us to precisely establish the localization of the Po_2 measurements in arterioles, venules, and the interstitium (20). The phosphorescence decay due to quenching at a specific Po_2 yields a single decay constant, and in vitro calibration has been demonstrated to be valid for in vivo measurements. Intravascular and perivascular Po_2 measurements were made in the arterioles studied, and intravascular Po_2 measurements were made in venules. Interstitial tissue Po_2 was measured in regions distant from visible underlying and adjacent vessels.

Tissue oxygen delivery and extraction. The microvascular methodology used in our studies allows a detailed analysis of oxygen supply in the tissue. Calculations of O_2 delivery, defined as the amount of oxygen delivered by the arterioles to the microcirculation per unit time normalized relative to baseline, and O_2 extraction, defined as the amount of oxygen released by blood to the tissue by the microcirculation per unit time normalized relative to baseline, were made using *Eqs. 1* and *2*:

$$O_2 \text{ delivery} = \{(RBC_{Hb} \times \gamma \times Sa_{RBC}\%) + (HbV_{Hb} \times \gamma \times Sa_{HbV}\%) + (1 - Hct) \times \alpha \times Pa_{O_2}\} \times Q \quad (1)$$

$$O_2 \text{ extraction} = \{[RBC_{Hb} \times \gamma \times S(a - v)_{RBC}\%] + [HbV_{Hb} \times \gamma \times S(a - v)_{HbV}\%] + (1 - Hct) \times \alpha \times P(a - v)_{O_2}\} \times Q \quad (2)$$

where RBC_{Hb} is the [Hb] in RBC (expressed in g/dl of blood), HbV_{Hb} is the [Hb] in HbV (expressed in g/dl of blood), γ is the oxygen-carrying capacity of Hb at 100% saturation (or 1.34 ml O_2/g Hb), Sa% indicates the arteriolar oxygen saturation of RBC or HbV, S(a-v)% indicates the arteriovenous difference in oxygen saturation of RBC or HbV, (1 − Hct) is the fractional plasma volume (and converts the equation from units per dl of plasma to per dl of blood), α is the solubility of oxygen in plasma and is equal to 3.14×10^{-3} ml O_2/dl plasma mmHg, Pa_{O_2} is the arteriolar partial pressure of oxygen, $P(a\text{-}v)_{O_2}$ is the arteriovenous difference in Po_2, and Q is the microvascular flow for each microvessel as a percentage of baseline. The oxygen dissociation curves were determined as described before. In this analysis, microvascular Hct was corrected according to the findings of Lipowsky and Firrell (10).

Experimental procedure. Baseline systemic, microvascular, and hemodynamic characterizations were performed before the start of the exchange. After each exchange and a stabilization period of 10 min, systemic and/or microvascular measurements were performed. Exchanges began every hour. After the *level 3* exchange transfusion, the same measurements were repeated, and then the Po_2 distribution was determined using phosphorescence-quenching microscopy (9). The duration of the experiment was 3–4 h.

Data analysis. Results are presented as means ± SD unless otherwise noted. All data are presented as absolute values and ratios relative to baseline values. A ratio of 1.0 signifies no change from baseline, whereas lower and higher ratios are indicative of changes proportionally higher or lower than baseline. The same vessels and functional capillary fields were followed so that direct comparisons to their baseline levels could be performed, allowing for more robust statistics for small sample populations. For repeated measurements, time-related changes were assessed by analysis of variance (ANOVA). Data within each group were analyzed using ANOVA for nonparametric repeated measurement, and when appropriate, post hoc analyses were performed with the Dunn's multiple comparison tests. For *level 3* exchange, groups were analyzed using one-way ANOVA, and post hoc analyses were performed with the Bonferroni post tests. All statistics were calculated using GraphPad Prism 4.01 (GraphPad Software, San Diego, CA). Changes were considered statistically significant if $P < 0.05$.

RESULTS

Exchange transfusion. Twenty-four animals (55–65 g body wt) entered into the exchange-transfusion (hemodilution) protocol, and all tolerated the experiment without any visible discomfort. Microvascular studies were completed in six preparations for each test material, namely, the *level 2* rHSA, HbV_8, and HbV_{29}. The data were analyzed using a model for computing oxygen delivery to the tissue at the microscopic level.

Hematological changes. The exchange-transfusion protocol resulted in a final Hct ranging from 11.0 ± 0.5 to 11.4 ± 0.6%. The HbV_8 and HbV_{29} groups had a final plasma Hb concentration of 2.1 ± 0.1 g/dl, which increased the total Hb concentration in blood (RBC + Hb in plasma) to 5.7 ± 0.2–0.3 g/dl after completion of the *level 3* exchange transfusion. Thus oxygen-carrying capacities at this level were similar to those found at *level 2*, where total blood Hb concentration was 5.7 ± 0.3 g/dl (Hct 18.1 ± 0.7) (Table 2).

Systemic and blood gas parameters. Changes in the systemic parameters are presented in Fig. 3. Mean arterial pressure was statistically lower for the extreme hemodilution tests with rHSA and the HbV_{29} group and attained the highest value with HbV_8 viscosity. Heart rate after hemodilution followed by exchange transfusion with the HbV solutions was ~10% higher than baseline at the *level 3* exchange. The slight increase in heart rate was not statistically different.

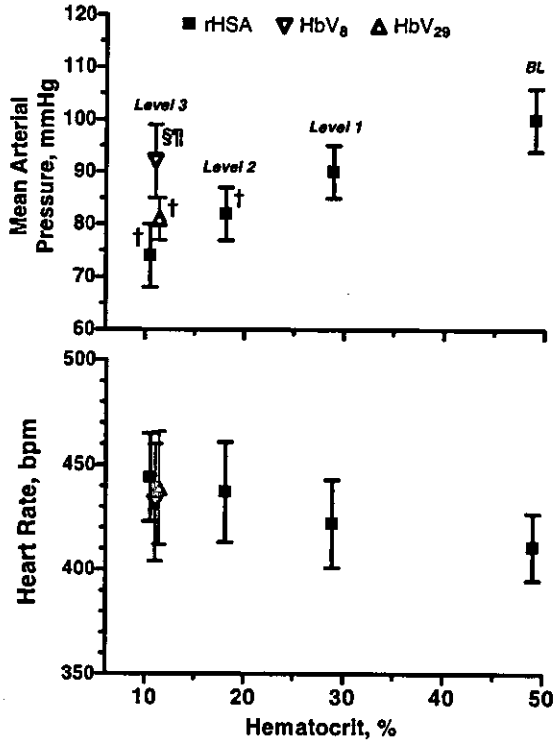

Fig. 3. Mean arterial blood pressure and heart rate [in beats/min (bpm)] at baseline (BL) (Hct 49%) and *level 1* (Hct 29%), *level 2* (Hct 18%), and *level 3* (Hct, 11%) hemodilutions. *Level 1* and *level 2* exchanges were performed with 5% rHSA as diluent. *Level 3* exchange was used to evaluate the oxygen transport of HbV_8 vs. HbV_{29} and rHSA. †$P < 0.05$ relative to baseline; §$P < 0.05$ relative to *level 3* rHSA; ¶$P < 0.05$, *level 3* with HbV_8 vs. *level 3* with HbV_{29}.

Analysis of arterial blood gases (Table 2) showed a statistical increase in Po_2 after hemodilution and exchange transfusion. Pa_{CO_2} was unchanged from baseline after hemodilution. Blood pH was not statistically changed. At *level 3* exchange, BE was positive and not statistically different between HbV groups, but it was negative and statistically different from baseline for the rHSA group ($P < 0.05$).

Colligative properties. Blood viscosities and COP after *level 3* exchange were sampled at 1 h and 10 min after completion

Table 2. *Laboratory parameters during exchange protocol*

		Level 1 Hemodilution	*Level 2* Hemodilution	*Level 3* Hemodilution		
	Baseline	rHSA	rHSA	rHSA	HbV_8	HbV_{29}
n	24	24	24	6	6	6
Hct, %	48.8±1.2	28.8±0.8*	18.1±0.7*	11.1±0.8*	11.0±0.5*	11.4±0.6*
Hb, g/dl						
Whole blood	14.8±0.4	9.0±0.5*	5.7±0.3*	3.7±0.4*	5.7±0.2*†	5.7±0.3*†
Plasma					2.1±0.1	2.1±0.1
Pa_{O_2}, mmHg	59.2±4.6	68.7±5.2	73.5±3.7*	87.5±7.0*	77.1±4.3*†	76.4±4.4*†
Pa_{CO_2}, mmHg	49.2±3.6	52.4±6.7	49.0±3.5	42.0±3.2*	53.0±3.9†‡	46.8±4.3
Arterial pH	7.35±0.02	7.35±0.03	7.37±0.03	7.38±0.04	7.35±0.03	7.36±0.03
HCO_3, mM	27.9±2.3	28.5±3.5	27.6±2.2	24.8±2.5	28.2±2.6	25.8±2.1
BE, mM	3.2±2.0	3.4±2.4	2.9±2.1	−0.2±1.9*	3.1±1.7†	1.0±2.0

Values are means ± SD. Baseline values include all animals in the study. No significant differences were detected between the baseline values of each group or between the values after *level 1* and *level 2* exchange before the exchange with test solutions. Hct, systemic hematocrit; Hb, hemoglobin content of blood; Pa_{O_2}, arterial partial O_2 pressure; Pa_{CO_2}, arterial partial pressure of CO_2; BE, base excess. *$P < 0.05$ compared with baseline; †$P < 0.05$ compared with *level 3* rHSA; ‡$P < 0.05$ compared with *level 3* HbV_8 to *level 3* HbV_{29}.

Table 3. *Rheological properties and COP*

Fluid	Blood Viscosity, cp	Plasma Viscosity, cp	COP, mmHg	*n*
Blood	4.2±0.7	1.2±0.1	17.6±0.7	6
Level 2 rHSA	2.0±0.2*	0.9±0.1	17.2±0.8	4
Level 3 rHSA	1.6±0.2*	0.9±0.1	17.4±1.1	5
Level 3 HbV_8	1.9±0.3*	1.0±0.1	17.3±0.8	6
Level 3 HbV_{29}	2.0±0.4*	1.0±0.1	17.8±1.0	5

Values are means ± SD; *n* = no. of animals studied. Viscosity was measured at a shear rate of 160 s^{-1} at 37°C. COP was measured at 27°C. Hct are presented in Table 2. *$P < 0.05$ compared with nondiluted blood.

of the exchange. Table 3 shows that blood viscosity ranges from 1.6 cp (plasma 0.9 cp) for rHSA to 2.0 cP (plasma 1.0 cp) for the HbV groups.

All test materials caused COP to maintain the value for normal blood for this species (5), namely, 17.6 ± 0.7 mmHg at 1 h after the last exchange, showing that introduction of bulk solutions into the circulation caused minor fluid shifts.

Microhemodynamics. After *level 3* exchange, arteriolar and venular diameters were not statistically different from baseline for any of the groups. Arteriolar flow velocities attained the highest value for the HbV_8 group, being 1.90 relative to baseline, which was statistically significant. The same effect was found in the venular microcirculation, where blood flow velocity was 2.20 relative to baseline. HbV_{29} exchange transfusion lowered both arteriolar and venular velocities relative to the values attained at the *level 2* exchange. However, venular velocity in this group was statistically significantly higher than in baseline. Notably, the *level 2* hemodilution with rHSA caused significantly higher blood flow velocities in the arteriolar and venular microcirculation (Fig. 4).

Combining data for the RBC flow velocity and diameter allowed calculation of the arteriolar and venular blood flows (Fig. 5). The results of this calculation showed that all exchanges caused blood flow to increase. Arteriolar and venular blood flows at *level 2* exchange with the use of rHSA were significantly higher than those at baseline. However, continuing hemodilution with this material to *level 3* exchange did not sustain the increase, and arteriolar and venular blood flow, although showing a tendency to remain elevated, were not statistically different from baseline values.

Level 3 exchange transfusion with HbV_8 and HbV_{29} caused blood flow to be significantly higher than baseline. Furthermore, the HbV_8 group showed consistently higher blood flows than the HbV_{29} group; however, the trend was not statistically significant.

Functional capillary density. The number of capillaries with RBC passage upon *level 3* hemodilution in the rHSA, HbV_8, and HbV_{29} groups was 62 ± 9, 76 ± 12, and 72 ± 13% of baseline, respectively. These values were statistically different from baseline but not statistically different with respect to each other (Fig. 6).

Microvascular oxygen distribution. Oxygen tension measured using phosphorescence microscopy after *level 3* exchange transfusion in the rHSA, HbV_8, and HbV_{29} groups showed that these materials produced virtually identical distributions of arteriolar microvascular P_{O_2} (arterioles averaged 49.5 mmHg), although HbV_8 tended to be higher (Fig. 7). The decrease of RBC from *level 2* to *level 3* did not decrease the arteriolar P_{O_2}. Venular P_{O_2} after *level 3* was significantly lower than at *level 2* exchange in all cases (rHSA, 7.2 ± 3.2 mmHg; HbV_8, 15.1 ± 3.7 mmHg; HbV_{29}, 9.6 ± 4.2 mmHg).

Tissue P_{O_2} values at *level 3* exchange were consistently lower than those at *level 2* exchange (20.1 ± 2.2 mmHg), with the difference being statistically significant. The highest was attained by the HbV_8 group, being 14.0 ± 2.2 mmHg. By comparison, tissue P_{O_2} for the HbV_{29} group was 9.2 ± 2.7 mmHg and for the rHSA group, 2.6 ± 1.4 mmHg, which was significantly lower compared with the HbV_8 and HbV_{29} groups (Fig. 7).

Oxygen delivery and extraction. Figure 8 shows the results of the analysis for delivery and release of oxygen by the

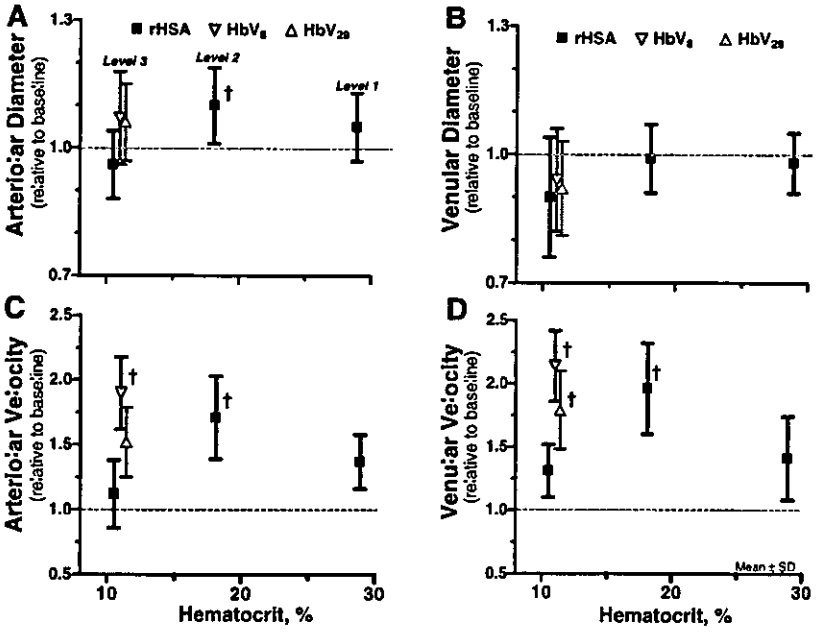

Fig. 4. Changes relative to baseline in arteriolar and venular hemodynamics at the *level 1*, *level 2*, and *level 3* exchanges. Dashed lines represent baseline level. †$P < 0.05$ relative to baseline. Arteriolar (*A*) and venular (*B*) diameters (μm, means ± SD, *n* = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 61.2 ± 10.2, *n* = 80; venules (V), 62.0 ± 12.3, *n* = 80. *Level 1* with rHSA: A, 65.6 ± 12.4; V, 61.1 ± 12.4. *Level 2* with rHSA: A, 66.1 ± 14.6; V, 64.3 ± 15.2. *Level 3* with rHSA: A, 63.7 ± 14.4, *n* = 20; V, 61.0 ± 16.7, *n* = 20. *Level 3* with HbV_8: A, 64.9 ± 15.7, *n* = 20; V, 64.6 ± 18.2, *n* = 20. *Level 3* with HbV_{29}: A, 66.1 ± 16.2, *n* = 20; V, 63.1 ± 18.0, *n* = 20. Arteriolar (*C*) and venular (*D*) RBC velocities (mm/s, means ± SD) in each animal group were as follows. Baseline: A, 4.9 ± 1.3; V, 1.7 ± 0.5. *Level 1* with rHSA: A, 6.3 ± 1.4; V, 2.0 ± 0.7. *Level 2* with rHSA: A, 7.9 ± 1.5; V, 2.6 ± 0.9. *Level 3* rHSA: A, 5.2 ± 2.0, *n* = 20; V, 1.9 ± 1.1, *n* = 20. *Level 3* with HbV_8: A, 7.2 ± 1.8, *n* = 20; V, 3.3 ± 1.0, *n* = 20. *Level 3* with HbV_{29}: A, 7.0 ± 1.7, *n* = 20; V, 3.0 ± 0.9, *n* = 20.

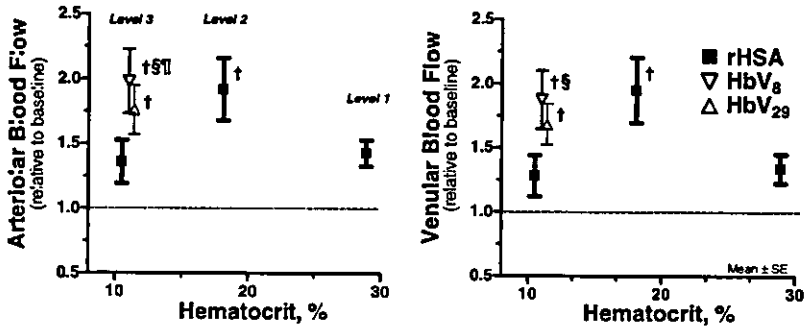

Fig. 5. Arteriolar and venular flow (nl/s, means ± SD, n = no. of vessels studied) in each animal group were as follows. Baseline: arterioles (A), 14.8 ± 7.1, n = 76; venules (V), 5.0 ± 2.9, n = 76. *Level 1* with rHSA: A, 21.9 ± 9.7; V, 5.8 ± 3.6. *Level 2* with rHSA: A, 27.2 ± 16.1; V, 8.3 ± 4.2. *Level 3* with rHSA: A, 16.9 ± 6.8, n = 20; V, 5.4 ± 4.8, n = 20. *Level 3* with HbV_8: A, 23.4 ± 8.1, n = 18; V, 9.9 ± 5.1, n = 18. *Level 3* with HbV_{29}: A, 21.0 ± 8.0, n = 18; V, 8.3 ± 5.2, n = 18.

microcirculation. It is apparent that exchanging RBC for HbV_8 maintains oxygen delivery to the tissue, whereas HbV_{29} reduces this by ~20%, and continued hemodilution with a non-oxygen-carrying material significantly depresses oxygen delivery to the tissue, reducing this to half of that attained at the *level 2* hemodilution.

DISCUSSION

The principal finding of this study is that under identical extreme hemodilution conditions, with the use of vesicles encapsulating Hb with normal P_{50} (HbV_{29} = 29 mmHg) and low P_{50} (HbV_8 = 8 mmHg), tissue Po_2 is statistically significantly higher when the high oxygen affinity material is used, namely, 14.0 ± 2.2 vs. 9.2 ± 2.7 mmHg. The significantly increased tissue Po_2 attained with HbV_8 appears to be due to a series of incremental improvements in microvascular and macrovascular hemodynamics comprising the increase of arteriolar blood flow and mean arterial blood pressure, which was significantly higher ($P < 0.05$) for HbV_8 than for HbV_{29}.

In the hemodilution procedures of this study, blood was exchanged with a rHSA solution as a colloidal plasma expander, which was the same suspending medium used for the Hb vesicles. Therefore, in these experiments, we can make a direct comparison between an oxygen-carrying and non-oxygen-carrying blood substitute, uncomplicated by the presence of additional materials. Our results show that the *level 2* hemodilution with rHSA leads to maintained functional capillary density and significantly improved arteriolar and venular blood flow, although somewhat lowered central blood pressure. The latter finding is not necessarily negative and may reflect a lowered overall peripheral vascular resistance due to the decrease of blood viscosity after hemodilution. The fact that microvascular flow is significantly increased indicates that the *level 2* hemodilution with rHSA provides the tissue with adequate microvascular perfusion and that this colloid is an adequate plasma expander.

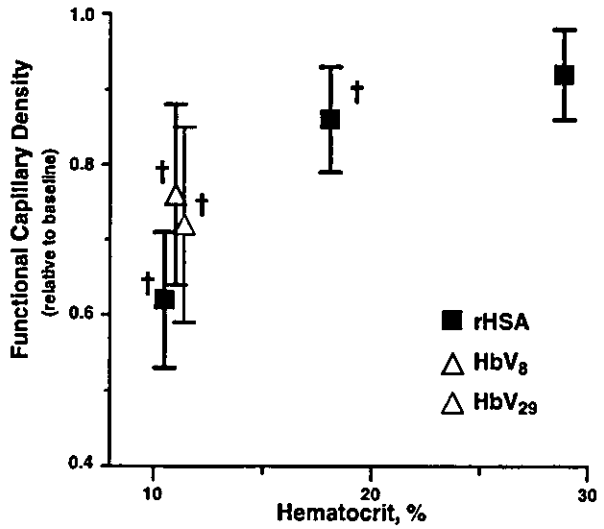

Fig. 6. Functional capillary density after the *level 1*, *level 2*, and *level 3* exchanges for the different test fluids. All values are relative to baseline levels. †$P < 0.05$ relative to baseline.

Average oxygen delivery and extraction were somewhat greater for HbV_8 than for HbV_{29}. These are calculated values and are not statistically significantly different; however, the same difference was found in all micro and macro parameters measured in this study.

The *level 2* hemodilution and the succeeding *level 3* hemodilution with either HbV_8 and HbV_{29} resulted in the same total Hb concentration in the circulation (5.7 and 5.8 g Hb/dl); however, oxygen delivery was lower with HbV_{29} and lowest with rHSA, as might be expected due to the low Hb content (3.7 g Hb/dl) in the absence of plasma Hb for the rHSA group. Therefore, because all groups had the same Hct at the *level 3* hemodilution, the sustained oxygen consumption and tissue Po_2 relative to the rHSA group clearly demonstrate that Hb vesicles release oxygen. However, the vesicles with the lowest P_{50} provide an oxygen delivery capacity identical to that of blood at *level 2* hemodilution, whereas vesicles with a high P_{50} lower oxygen delivery at the microcirculatory level, an effect probably caused by the decreased blood flow associated with HbV_{29}.

The differences in tissue Po_2, mean arterial blood pressure, and arteriolar blood flow between HbV_8 and HbV_{29} show that in designing a blood substitute, it is not sufficient to provide adequate oxygen-carrying capacity. Once a suitable oxygen carrier is available, it also must be able to maintain or enhance other circulatory transport parameters, particularly flow. The Hb vesicles used in this study are vasoinactive, and the difference in P_{50} appears to be a factor in improving flow condition that is not related to vasoactivity. An explanation for this may be related to the inherent variability of tissue Po_2 shown in this and other studies (4, 22), which may be enhanced in extreme hemodilution. This variability determines that if average tissue Po_2 is low, portions of the tissue may become anoxic. Introducing a small quantity of a low-P_{50} Hb oxygen carrier into the circulation will deliver oxygen only to those parts of the tissue