with the components of blood and vasculature during circulation [9]. In comparison with some acellular Hb modifications, the Hb encapsulation in vesicles suppresses hypertension induced by vasoconstriction, a theory that is suggested to be due to the high affinity of Hb with nitric oxide and carbon monoxide as vasorelaxation factors [10,11]. Moreover, the surface modification of HbV with polyethylene glycol (PEG) chains not only prolongs the circulation half-life [12] but also prevents the intervesicular aggregation and guarantees the homogeneous dispersion in the plasma phase that provides a prompt blood flow in the microcirculation and the resulting sufficient tissue oxygenation [13,14].

According to the clinical conditions HbVs are supposed to be applied for, the organism is faced with the metabolism of a large amount of both Hb and lipids, because the dose rate of HbV is significantly large. The HbV particles, as well as phospholipid vesicles, infused in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [4,15]. In a previous report, we clarified by the histopathological studies of rats receiving 20 ml/kg of HbV infusion that the HbV particles were captured and metabolized within 7 days in RES mainly in the spleen and liver [16]. Transmission electron microscopy provided a clear image of the HbV particles in the phagosomes 1 day after infusion, but they disappeared within 7 days. Staining with the anti-human Hb antibody, Berlin blue, and hematoxylin/eosin showed prompt metabolism of Hb molecules with no morphological changes in the liver and spleen. The phagocytic activity decreased and then transiently increased, but tended to return to the original level. From these studies, we did not see any irreversible damage to the organs.

Serum laboratory tests are the most common diagnostic tools to monitor organ functions clinically. However, both the PEG-modified HbV particles and the chemically modified Hb solutions remained in the plasma even after usual centrifugation to remove RBC, showing significant interference effects due to the light absorption by Hb and light scattering by the particles. These interference effects hindered the accurate evaluation of plasma laboratory tests and have been regarded as a serious issue for the development of HBOCs [17,18]. However, quite recently we have clarified by an in vitro experiment that the simple removal of PEG-modified HbV as a precipitate by ultracentrifugation (50,000 *g*, 20 min) or by conventional centrifugation in the presence of a high-molecular-weight dextran diminished most of the interference effects [19]. Using this simple procedure, we aimed to evaluate the safety of HbV by the laboratory tests of plasma after bolus intravenous infusion of HbV at a rate of 20 ml/kg, the same experimental model as in the previous study [16].

2. Materials and methods

2.1. Preparation of PEG-modified HbV

The PEG-modified HbV was prepared in a sterile condition as previously reported in the literature [10, 20–22]. Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Society of Red Cross, Japan (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP, Sigma) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. 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 Chem. Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF Co., Tokyo, Japan, 0.3 mol% of the total lipid). The HbCO solution and the lipid powder were mixed and stirred for 12 h at 4°C. The resulting multilamellar vesicles were extruded through membrane filters with a final filter pore size of 0.22 μm. Thus prepared PEG-modified HbV was suspended in saline at the Hb concentration of 10 g/dl, and filtrated (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], <3%; [HbCO], <2%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was precisely measured by modified *Limulus* Amebocyte lysate gel-clotting analysis that has been developed by our group recently, and confirmed that the endotoxin content was less than 0.1 endotoxin unit/ml [23].

2.2. HbV infusion and procedure for the plasma laboratory tests

All animal studies were approved by the Animal Subject Committee of Keio University School of Medicine and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985). The experiments were carried out using 40 male Wistar rats (200–210 g, Saitama Experimental Animals, Kawagoe, Japan). They were anesthetized with diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg ($n = 5$ for every time point). Ten rats were used to obtain the control values. 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.

After 8 h, and 1, 2, 3, and 7 days, the rats were anesthetized with 1.5% sevoflurene inhalation (Maruishi Pharm. Co., Osaka, Japan) using a vaporizer (Model

TK-4 Biomachinery, Kimura Med., Tokyo). Polyethylene tubes (PE-50, Natsume Co., Tokyo) were implanted in the carotid artery for withdrawing blood into heparinized syringes for the Hct, HbV concentration, and plasma laboratory tests. The animals were finally laparotomized and sacrificed with acute bleeding from the abdominal aorta and the liver and spleen were obtained for weight measurements. The control rats received the same procedure for the measurements.

A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma which was turbid and red/brown colored due to the presence of PEG-modified HbV particles especially in the samples taken at 8 h, 1 and 2 days after infusion. The plasma was ultracentrifuged (50,000 *g*, 20 min) to remove the HbV particles. The obtained transparent plasma specimens were stored at −80°C until the laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ -glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), cholinesterase (ChE), leucin amino peptidase (LAP), creatine phosphokinase (CPK), amylase, lipase, total cholesterol (Total-Chol.), cholesteryl ester (Chol.Ester), free cholesterol (Free-Chol.), HDL-cholesterol (HDL-Chol.), β-lipoprotein, triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, uric acid (UA), blood urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate (IP), and Fe^{3+}. In our previous study, it was confirmed that the concentrations of the plasma components in terms of the above analytes did not change after the ultracentrifugation at 50,000 *g* for 20 min [19]. Since rat albumin is slightly insensitive to the bromcresol green method, the values were corrected according to Takano et al. [24].

2.3. Histopathological examination of pancreas

After sacrificing the animals by acute bleeding from the abdominal aorta, the pancreas was resected for a histopathological study. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chem. Co., Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

2.4. Data analysis

Differences between the control and a treatment group were analyzed using a one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) test. The changes were considered statistically significant if $p<0.05$.

3. Results

All the rats receiving the bolus infusion of HbV at a dose rate of 20 ml/kg tolerated the infusion and survived until intentional sacrifice. There was no noticeable change in appearance such as piloerection.

3.1. Hct and circulation persistence of HbV

The control Hct was 42±1%, and it decreased slightly to 40±1% at 1 day after HbV infusion. The estimated Hb concentration of HbV in plasma just after infusion was about 6 g/dl, and it gradually decreased to 4.4±0.3 g/dl at 8 h, 1.9±0.2 g/dl at 1 day, 1.3±0.1 g/dl at 2 days, and 0.8±0.01 g/dl at 3 days (Fig. 1). At 7 days, HbV was not detected at all in the plasma phase.

3.2. Spleen and liver weights

The changes in the spleen and liver weights were expressed as percents of the body weight (Fig. 1). The liver weight ratio (control, 4.81±0.17%) showed a significant increase 1 day after the infusion (5.29±0.27%, $p<0.01$), and then it returned to the original level at 2 days. Spleen weight ratio significantly increased from 0.32±0.05% to 0.66±0.06% 3 days after the infusion ($p<0.01$), however, it was reduced to 0.41±0.02% at 7 days.

3.3. Plasma laboratory tests

The plasma fraction after centrifugation of the blood sample for 3 days after the HbV infusion was turbid due to the presence of PEG-modified HbV. However, ultracentrifugation of the plasma produced transparent and light-yellow plasma phase and PEG-modified HbV was precipitated at the bottom in a tube. There was no sign of the presence of Hb in the supernatant, indicating that there was no hemolysis of both RBC and HbV.

As for the analytes that reflect the liver function, the total protein (control, 5.2±0.1 g/dl) and albumin (2.46±0.06 g/dl) slightly decreased to, e.g., 4.9±0.2 and 2.11±0.10 g/dl, respectively, with statistically significant differences ($p<0.01$) for 3 days after the HbV infusion (Fig. 2). They tended to return to its original level at 7 days ($p<0.05$). AST (control, 60±7 U/l) decreased to 46±3 U/l ($p<0.05$) and returned to the original level at 7 days. ALT (control, 32±5 U/l) only slightly increased to 40±8 U/l 1 day after the HbV infusion ($p<0.01$), but it returned to its original level 2 days after the infusion. LDH (control, 150±60 U/l) did not change significantly. ALP (control, 1265±231 U/l) decreased at 2 days (812±149 U/l) and 3 days (872±98 U/l) ($p<0.01$), but it returned to the control level at 7 days. γ-GPT (control, 1.6 U/l) and LAP (31±1 U/l) showed significant but minimal reductions

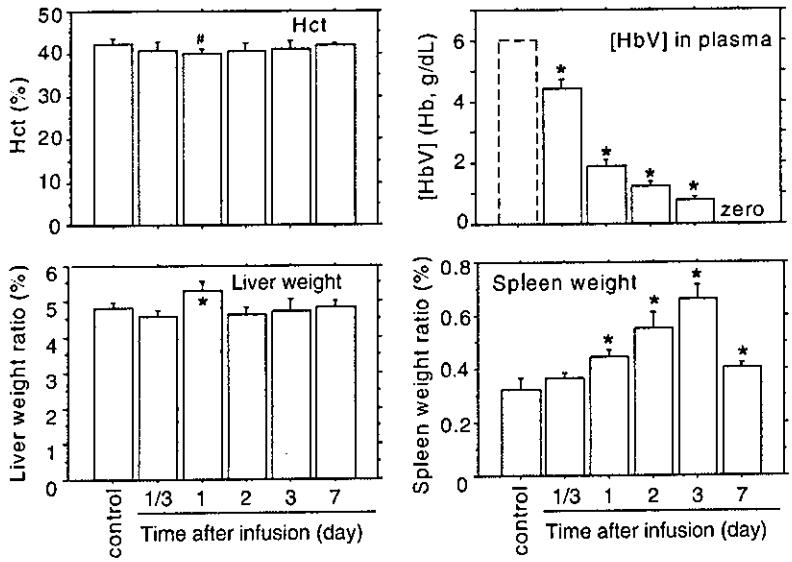

Fig. 1. Changes in hematocrit, concentration of HbV in plasma, and spleen and liver weights after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. The control value of [HbV] is the estimated concentration of HbV immediately after the infusion and expressed as with a dashed line. The spleen and liver weights are expressed as the ratio to the body weight (%).

($p<0.05$). ChE (control, 76 ± 18 U/l) did not show a noticeable change. Plasma total bilirubin (≤0.1 mg/dl) and Fe^{3+} showed some reductions but were maintained at a low level for 7 days in spite of the metabolism of a large amount of Hb.

CRE (control, 0.3 mg/dl) was maintained at a low level for 7 days. BUN (control, 16 ± 3 mg/dl) showed a slight increase at 7 days (21 ± 3 mg/dl) (Fig. 3). UA (control, 0.47 ± 0.19 mg/dl) increased to 0.70 ± 0.16 mg/dl at 3 days, however, it returned to a non significant level at 7 days. Amylase (control, 1613 ± 74 U/l) significantly decreased for 3 days after the infusion ($p<0.01$), but returned to its original level at 7 days. Lipase (control, 9 ± 1 U/l) showed significant increases ($p<0.01$) after the HbV infusion, and it tended to decrease after 3 days, and was reduced to a non-significant level at 7 days. CPK (control, 304 ± 116 U/l) decreased at 7 days ($p<0.05$), but did not show a noticeable increase during the experiment. As for the electrolyte concentrations, K^{+}, Ca^{2+}, and IP did not show any significant changes.

The most consistent changes were seen in the lipid components (Fig. 4). Total-Chol. (control, 73 ± 7 mg/dl), Free-Chol. (18 ± 2 mg/dl), Chol.Ester (59 ± 8 mg/dl), and HDL-Chol. (32 ± 4 mg/dl) showed significant increases and maximum values at 2 days ($p<0.01$). Free-Chol. increased to 39 ± 4 mg/dl, about twice the control value. However, it tended to decrease at 3 days, and returned to its control level at 7 days. β-Lipoprotein (control, 110 ± 42 mg/dl) slightly increased at 1 day (160 ± 33 mg/dl), but returned to its original level at 3 days. TG (control, 64.4 mg/dl) significantly decreased to 12.4 mg/dl at 2 days ($p<0.01$), but tended to increase to its original level at 7 days. Phospholipid (control, 132 ± 8 mg/dl) significantly increased to 150 ± 9 mg/dl at 1 day ($p<0.01$), and then returned to the original level at 3 days.

3.4. Histopathological examination of pancreas

The histology of pancreatic tissue 2 days after the infusion of HbV is shown in Fig. 5. There was no significant morphological change in spite of the increment of the pancreatic lipase activity.

4. Discussion

The clinical indications for the use of the HbV suspension as an artificial O_2 carrying fluid are estimated to be mainly preoperative or perioperative hemodilution, or resuscitation from hemorrhagic shock in emergency situations [25], both of which result in exchanging more than 20% of the original blood with the HbV suspension. Thus, the dose amount is extremely greater than that of stealth liposomes for drug delivery systems. HbV particles in the blood stream are finally captured by RES in the same manner as the conventional phospholipid vesicles [15]. In a previous study, we confirmed by the histopathological examination in a rat model that HbV particles were captured in the phagosomes of liver Kupffer cells and spleen macrophages without tissue damage, and they had completely disappeared within 7 days [16]. The transient splenomegaly and hepatomegaly in Fig. 1 seemed associated with the entrapment of HbV. The total weight change of

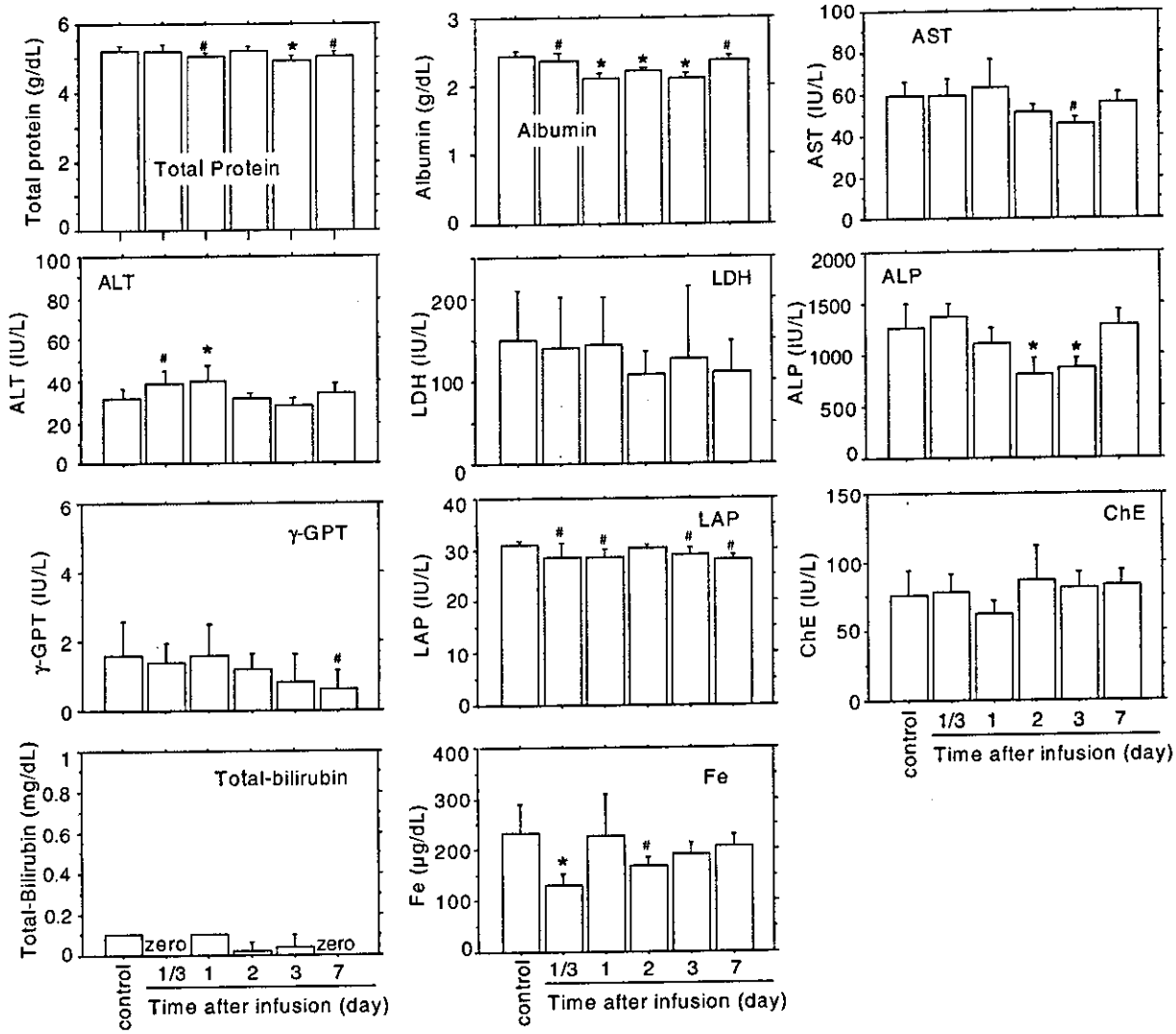

Fig. 2. Plasma laboratory tests representing the liver function and metabolism of Hb after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), leucin amino peptidase (LAP), cholinesterase (ChE).

these organs is 0.8% of the body weight (1600 mg for 200 g body weight), which should correspond to not only the accumulated HbV (635 mg for 20 ml/kg) but also to the increased amount of phagocytic or parenchymal cells and/or RBC. The organ weight ratios tended to return to their original levels as HbV disappeared from the blood stream, and there was no deteriorative sign of morphological change in the main organs such as the liver, spleen, lung, kidney, and heart. To confirm the safety more in detail, we analyzed for the first time, the plasma laboratory tests on 29 analytes without any interference effect of the PEG-modified HbV simply by removing it from plasma by ultracentrifugation [19].

Our results indicated no irreversible sign of organ damage after the bolus infusion of HbV at a dose rate of 20 ml/kg (cf. whole blood = 56 ml/kg). Especially, liver is one of the main organs of the trapping and metabolism of HbV. However, we did not see an increase in the physiological meaning of the parameters representing the liver function. As for the parameters representing the renal function, there were slight changes in CRE, BUN, and UA without any physiological meanings. CPK did not significantly change, indicating that the intactness of the cardiac function and skeletal muscular function should be preserved.

Amylase and lipase that represent pancreatic function showed slight changes. The amylase activity slightly decreased while the lipase activity significantly increased from 9±1 IU/l at control to 30±9 IU/l at 2 days. The lipase activity was measured by an enzymatic method that was specific for pancreatic lipase. Therefore, the increment should not be attributed to the hepatic or

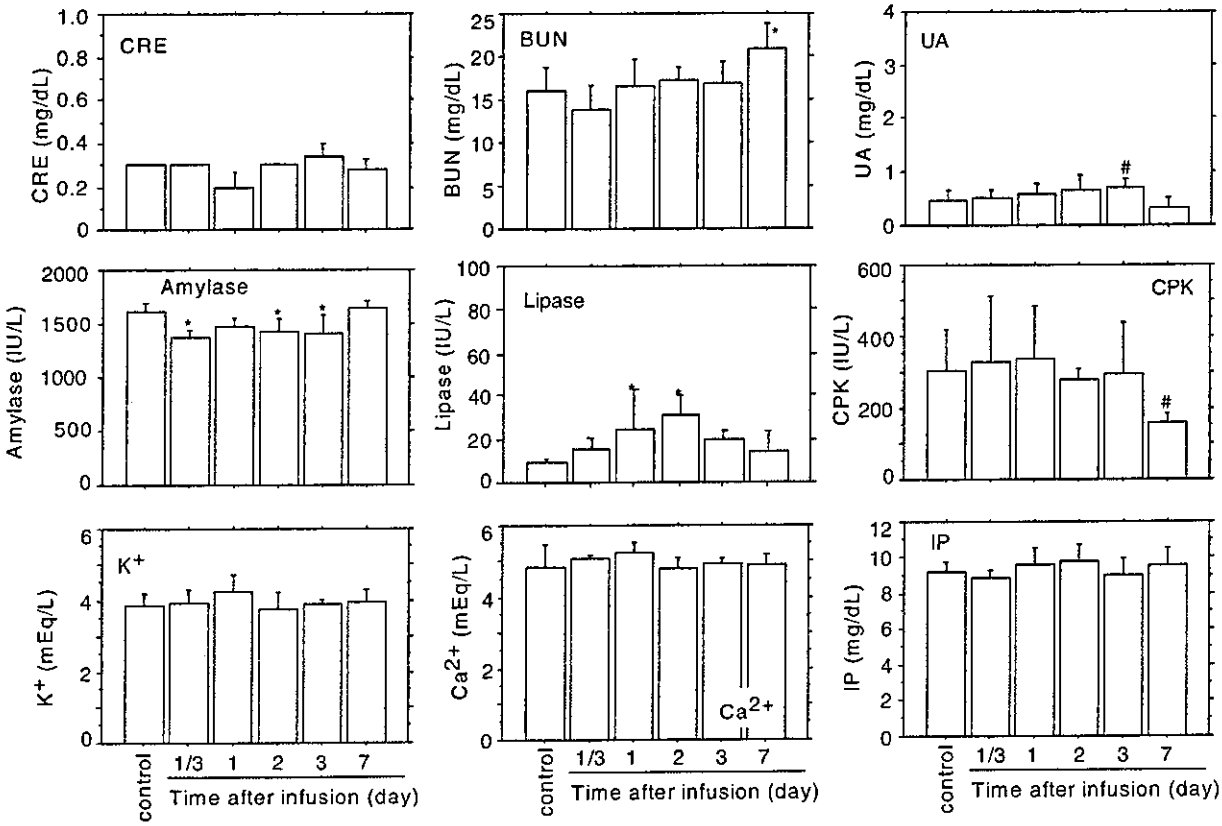

Fig. 3. Plasma laboratory tests representing renal, pancreatic and myocardial function, and electrolytes after infusion of HbV (20 ml/kg). The values are mean±SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), creatine phosphokinase (CPK), inorganic phosphate (IP).

lipoprotein lipase. However, this level of increment was significantly smaller than the reported value for the Wistar rats of pancreatitis. Hofbauer et al. [26] reported that acute necrotising pancreatitis increased lipase activity from 10 to 475–5430 IU/l. It was reported that the injection of liposome amphotericin B raised the serum lipase activity, and one possible reason was speculated to be the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes [27], because pancreatic lipase hydrolyze not only TG but also phosphatidylcholine [28]. This speculation was also supported by our results that the profiles of the transient increases in the lipid components coincided with that of lipase, but not with amylase. The cause of this modification is not clear at the present time. Histopathological analysis showed no significant pathological change in the pancreas. However, the pancreatic function should carefully be monitored in the ongoing safety studies.

Significant and consistent increases were seen in the lipid components with maximum at 1 or 2 days. They should be derived from the HbV particles because they contain a large amount of cholesterol (ca. 1200 mg/dl) and DPPC (1840 mg/dl) in the infused suspension ([Hb] = 10 g/dl). The gradual increases in cholesterols by 2 days after infusion and no Hb release from HbV in the plasma indicate that they should be liberated from RES after HbV are captured by RES and destroyed in the phagosomes. This is also supported by the fact that the maximum concentrations were seen at 2 days when the HbV in the plasma had mostly disappeared from the blood. It has been reported that the infused lipid components of the phospholipid vesicles are trapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in the bile and in the exhaled air [29–31]. Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV is so large that it cannot diffuse across the fenestrated endothelium into the space of Disse [11]. Cholesterol from HbV should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells [32], and then excreted in the bile after entrapment of the corresponding lipoprotein by the hepatocytes [33]. We speculate that the main components of the lipid bilayer membrane of HbV, the phospholipids and cholesterol, would gradually be redistributed

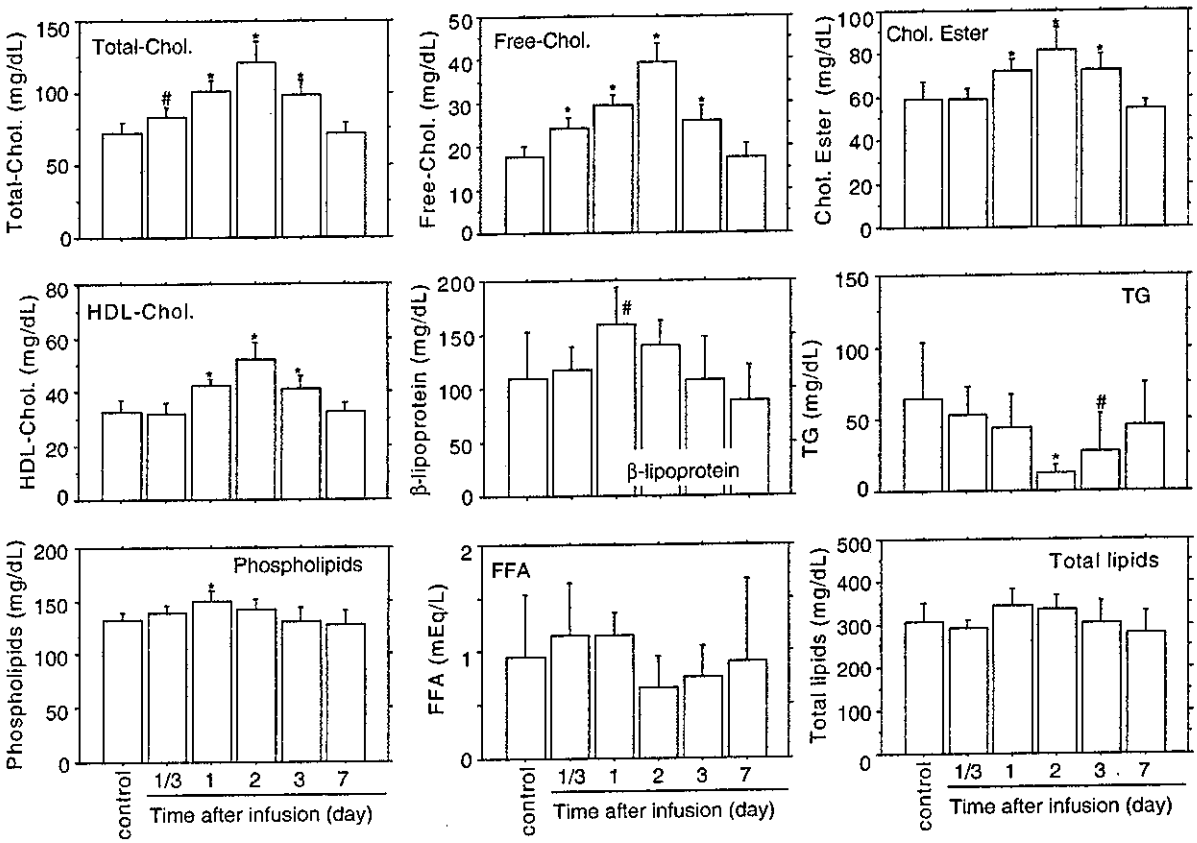

Fig. 4. Plasma laboratory tests representing lipid metabolism after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: total cholesterol (Total-Chol.), free cholesterol (Free-Chol.), cholesteryl ester (Chol.Ester), HDL-cholesterol (HDL-Chol.), triglyceride (TG), free fatty acid (FFA).

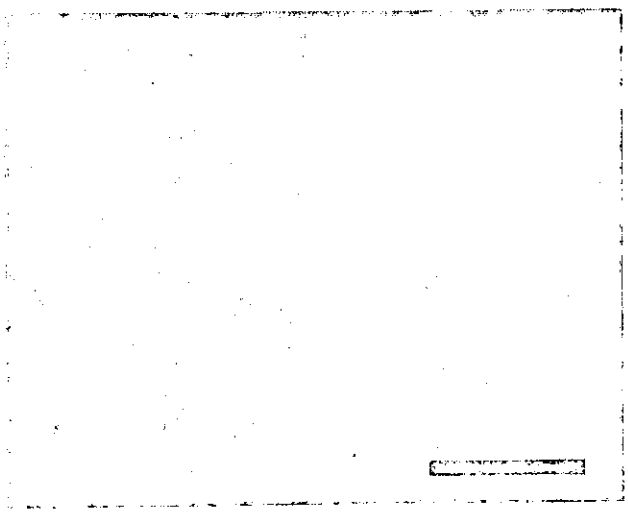

Fig. 5. Histology of pancreas 2 days after the infusion of HbV (20 ml/kg). Bar indicates 100 μm (HE stain).

or metabolized in the same manner. However, a precise pharmacokinetic study is necessary using radiolabeled materials to demonstrate the metabolic and excretory routes of the lipids. Transient, but significant increases in the lipid components raise the necessity of a further study to clarify the influence of a large dose of HbV especially on a lipemic model.

During the metabolism of Hb, there should be a release of bilirubin and iron. However, they did not increase for 7 days. In a previous study, the anti-human Hb antibody staining was effective for detecting the special and temporal distribution of human Hb of HbV both in the spleen and liver [16], and we made it clear that human Hb disappeared within 7 days. The released heme from Hb in HbV may probably be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells in the liver and in the spleen [11,34]. Bilirubin should be excreted in the bile as a normal pathway, and there should be no obstruction or stasis of bile in the biliary tree. Berlin blue staining revealed the presence of hemosiderin 3 and 7 days after HbV infusion, and it disappeared after 14 days [16]. A similar observation was reported for a polymerized Hb that was captured by the Kupffer cells while showing subsequent hemosiderin formation [35]. Normally, iron from a heme is stored in the ferritin molecule [36]. Ferritin in the lysosomal membrane may form paracrystalline structures and

eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Both ferritin and hemosiderin release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation [37,38]. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert [39]. Plasma iron, mostly bound to transferrin, remained constant after HbV infusion. The iron concentration should be coordinately regulated through the "iron regulatory proteins" that sense the levels of iron for hematopoiesis and metabolic needs [40], and the excess amount of iron should be stored in an insoluble and less toxic form as hemosiderin. Together with the time course of the histopathological changes, the results of the plasma laboratory tests indicate that the metabolism of heme and the recycling or excretion of iron molecule is within the physiological capacity and suggested to be on the physiological pathway that has been well characterized for the metabolism of senescent RBC [41].

5. Conclusion

In this study, the plasma laboratory tests after the infusion of HbV (20 ml/kg) did not demonstrate an irreversible sign for a deteriorative damage to the organs. Plasma bilirubin and iron, which were considered to be released during the metabolism of the Hb molecule, did not increase during the observation period. This may be due to the moderate rate of Hb metabolism in RES after the entrapment of HbV with a moderate length of circulation time. The lipid components significantly increased at 2 or 3 days after infusion. These may be derived from the membrane component of HbV entrapped in RES. The complete normalization of the lipid components indicates that they are metabolized in a normal metabolic and/or recycling pathway. The precise biodistribution and fate of the components should be confirmed by a radioisotope technique. Our results have demonstrated the safety of HbV using only healthy rats, while rats in hemorrhagic shock, septic shock, or lipemia have to be tested in the ongoing safety studies. It should also be emphasized that the data cannot be extrapolated to large animals or humans, which may react differently to such a large dose of HbV.

Acknowledgements

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Hemorrhagic Shock Resuscitation With an Artificial Oxygen Carrier, Hemoglobin Vesicle, Maintains Intestinal Perfusion and Suppresses the Increase in Plasma Tumor Necrosis Factor-α

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It is known that damage to the intestinal mucosa followed by systemic inflammatory response is one of the leading causes of shock related morbidity and mortality. In this study, we examined the ability of an artificial oxygen carrier hemoglobin vesicle (HbV) to sustain systemic and intestinal perfusion during hemorrhagic shock. In rabbits, hemorrhagic shock (40% of the estimated blood volume) was resuscitated with 5% albumin (alb group), HbV suspended in 5% albumin (HbValb group), or washed red blood cells suspended in 5% albumin (RBCalb group). Plasma tumor necrosis factor (TNF)-α level was measured in rats under the same experimental protocol. No significant intergroup differences were seen in systemic hemodynamics. In contrast, parameters of intestinal perfusion significantly deteriorated in the alb group but were equally well sustained in the HbValb and RBCalb groups. Also, a significant increase in plasma TNF-α level was seen in the alb group but not in the RBCalb or HbValb groups. These results indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. *ASAIO Journal* 2004; 50:458–463.

Blood replacement is the basic therapeutic modality when a considerable amount of blood is lost because of trauma or major surgery. Despite the recent progress in transfusion medicine, enormous investments are still necessary to establish and sustain the systems from blood donation to transfusion. Donated blood inspections to avoid the side effects of homologous blood transfusion, such as transfusion associated infectious disease, alloimmunization, and graft *versus* host diseases are still essential.[1,2] To overcome these problems associated with transfusion, development of artificial blood substitutes is important. To this end, we have developed several types of artificial oxygen carriers and have evaluated the efficacy of these compounds in various animal models.[1] Among these compounds, hemoglobin vesicle (HbV), a form of liposome encapsulated hemoglobin, is rapidly approaching clinical trials. The cellular structure of HbV, similar to red blood cells, shields all of the physiologic effects of acellular Hb solutions.[3–5] We have studied the oxygen transporting capabilities of HbV, using several exchange transfusion and hemorrhagic shock models.[6–10] In these studies, we have shown that HbV effectively restores the systemic circulation in hemorrhagic shock.

It is known that gastrointestinal perfusion is compromised at a relatively early stage in hypovolemic shock to sustain the systemic circulation to other vital organs.[11] This, however, causes damage to the intestinal mucosa followed by systemic inflammatory response syndrome (SIRS) or sepsis, which is one of the leading causes of shock related morbidity and mortality.[12,13] In the present study, we examine the ability of HbV to sustain not only systemic but also intestinal perfusion to further evaluate the efficacy of HbV in hemorrhagic shock.

Materials and Methods

Animal Care

The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of Keio University, School of Medicine. It also complies with Guidelines for the Care and Use of Laboratory Animals of Keio University, School of Medicine. All rabbits and rats were housed in groups of two in standard cages and were provided with food and water in a temperature controlled room on a 12 hour dark/light cycle.

Preparation of Hemoglobin Vesicle Suspended in 5% Albumin

HbV suspension was prepared in a similar manner as previously reported in the literature.[14,15] In brief, a purified and concentrated human hemoglobin solution (40 g/dl) was obtained from outdated red blood cells.[16] Added to this purified hemoglobin solution were pyridoxal 5'-phosphate (18 mM, Merck Co., Frankfurter, Germany) as an allosteric effector and homocysteine (Aldrich Co., Milwaukee, WI) as a reductant of methemoglobin. The lipid bilayer of HbV was composed of Presome PPG-I (Nippon Fine Chem. Co., Osaka, Japan) containing 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, (DPPC), cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phos-

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Table 1. Physicochemical Properties of PEG Modified HbV Suspended in Human Serum Albumin

Hb (g/dl)	10
Lipid (g/dl)	6.2
Hb/lipid (g/g)	1.61
Diameter (nm)	251 ± 87
P_{50} (torr)	32
Hill number	2.2
Viscosity (cP at 358 s^{-1})	3.7
HbCO (%)	2
MetHb (%)	3

PEG,.

phatidylglycerol (DPPG), which were purchased from Nippon Fine Chem. Co. (Osaka, Japan), and α-tocopherol was added to these at the composition so that the molar ratios for DPPC: cholesterol:DPPG: α-tocopherol became 5:5:1:0.1. The surface of the HbV was modified with poly(ethylene glycol) (Mw: 5 kDa, 0.3 mol% of the lipids in the outer surface of vesicles) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (Sunbright DSPE-50H, H-form, NOF Co., Tokyo, Japan). HbVs were suspended in 5% human serum albumin (alb) containing 160 mEq/L sodium and 107 mEq/L chloride (Albumin 5%-cutter, Bayer) and filtered through sterilizable filters (Dismic, Toyo Roshi Co., Tokyo, Japan, pore size: 0.45 micrometer). The whole procedure was performed at temperatures below 10°C in a sterile environment.

The properties of HbV suspended in alb (HbValb) are summarized in **Table 1**. The amount of oxygen release was calculated to be 6.2/100 ml. This is close to 7.0/100 ml of human blood (hemoglobin concentration 15 g/dl) because of, theoretically, the increased oxygen transporting efficiency (the difference in oxygen saturation between 40 and 110 mm Hg PO_2) of HbV compared with human red blood cells (37% to 28%, respectively).

Preparation of Washed Rabbit (Rat) Red Blood Cells Suspended in 5% Albumin

Blood samples were withdrawn from rabbits/rats into heparinized syringes and centrifuged to obtain a red blood cell concentrate. This was washed twice to remove plasma components and buffy coat by resuspension in 5% human serum albumin and centrifugation (4,300 rpm, 10 min). The hemoglobin concentration was adjusted to 10 g/dl, equivalent to that of HbValb.

Hemorrhagic Shock Resuscitation

Animal preparation was performed as follows (**Figure 1**). Male Japanese white rabbits (3.0 ± 0.4 kg) were anesthetized with intramuscular injection of ketamine hydrochloride (50 mg/kg) and intravenous injection of pentobarbital sodium (20 mg/kg) through the marginal ear vein. The body temperatures of the animals were maintained between 36 and 37°C by a heating lamp during the experiment.

Tracheostomy tubes were placed to secure the airway. The animals breathed spontaneously during the experiment. A polyethylene tube (outer diameter 1.7 mm, ATOM Japan) was introduced into the right carotid artery for blood withdrawal and connected to a pressure transducer (Polygraph System,

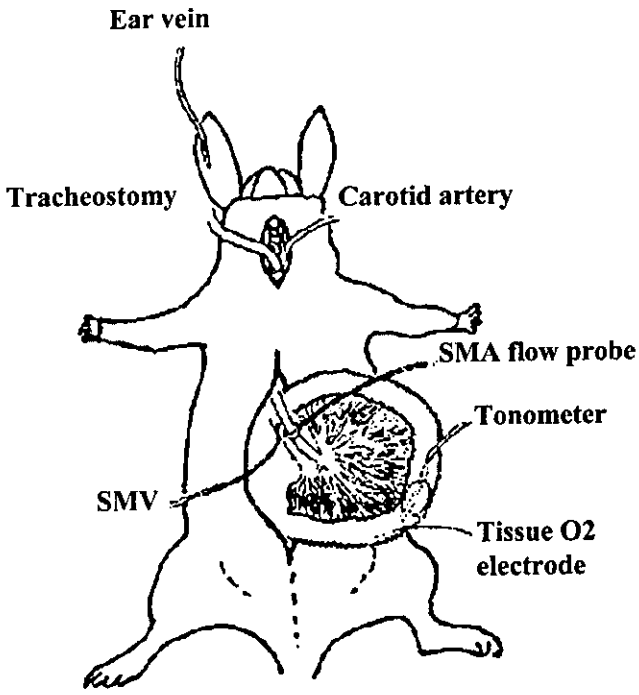

Figure 1. Schematic representation of the shock resuscitation experiment in the rabbit is shown. SMA, superior mesenteric artery; SMV, superior mesenteric vein.

Nihon Koden, Tokyo Japan) for continuous mean arterial pressure (MAP) monitoring. A median abdominal incision was made, and the superior mesenteric artery (SMA) was identified and dissected from surrounding tissue close to its origin from the aorta. A 2 mm ultrasonic flow probe (20 MHz, Crystal Biotech, Hopkinton, MA) was placed around the root of SMA and connected to a blood flow meter for measurement of SMA flow and heart rate. A small vein in the mesentery was ligated distally and cannulated with polyethylene catheter (PE-20). The catheter was advanced 5–10 cm proximally until the tip was located in the superior mesentric vein (SMV) for sampling of venous blood. For arterial and venous blood gas measurements, Corning 170 pH/blood gas analyzer (Corning Medical, Medfield, MA) was used. Hemoglobin concentration was determined by hemoglobin analyzer, Sysmex E-400 (Toa Medical Electronics Co, LTD, Kobe, Japan).

A sigmoid tonometor (Tonomiter Tonometorics) was positioned in the duodenum 2–3 cm from the pylorus for intestinal mucosal pH (pHi) measurements. The pHi was determined from partial carbon dioxide pressure (PCO_2) in the tonometer saline, the bicarbonate concentration, and the Henderson-Hasselbalch equation (I):

$$pHi = 6.1 + \log_{10}(HCO_3^-/(0.22 \cdot PCO_2 \cdot k)) \quad (1)$$

A needle type polarographic oxygen electrode (Intermedical, Tokyo, Japan) was inserted into the submucosa of the small intestine for continuous intestinal submucosal tissue oxygen tension measurements.

Approximately 20 minutes was allowed for MAP, SMA blood flow, and tissue oxygen tension measurements to stabilize. Hemorrhagic shock was induced by withdrawal of 40% of the estimated total blood volume of the rabbit from the right

carotid artery at a rate of 10 ml/min (3 ml/kg/min). Approximately 10 minutes after bleeding, they were infused with the lost volume *via* the marginal ear vein at the same rate with 5% albumin (alb group, n = 6), HbValb (HbValb group, n = 6), or washed rabbit red blood cell (RBCalb group, n = 6). This procedure was repeated twice. Arterial (carotid artery) and SMV blood samples were drawn before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30); pHi was measured at BASAL, BL2, IN2, and AFTER30.

Histologic Examination

After completion of the experiment, the animals were killed by pentobarbital overdose. The heart, lung, kidney, liver, spleen, and small intestine were removed and fixed in 10% formalin. The tissues were embedded in paraffin, and the sections were stained with hematoxylin and eosin for light microscopic examinations.

Tumor Necrosis Factor-α Measurements

Male Wistar rats (364 ± 15 g) were used for the experiment. They were anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg). A longitudinal midline ventral cervical incision was made, and catheters (PE-20 tubing, outer diameter 0.8 mm, inner diameter 0.5 mm) were introduced into the right jugular vein for infusion and into the right common carotid artery for blood withdrawal. Shock resuscitation was performed following the protocol in the rabbit. Forty percent of the estimated total blood volume was drawn from the right carotid artery at a rate of 1 ml/min (3 ml/kg/min). After bleeding, they were infused *via* the jugular vein with the same rate and volume of 5% albumin (n = 6), HbValb (n = 6), or washed rat red blood cell (n = 6). This procedure was repeated twice. Thirty minutes after the second infusion, corresponding to AFTER30, blood was sampled from the carotid artery. After centrifuging the blood at 4,300 rpm for 10 minutes, the plasma component was separated and stored at −80°C until measurement. TNF-α was measured by enzyme linked immunosolvent assay (ELISA) using Genzyme-Technic rat TNF-α determination kit.

Data Analysis

Data are shown as mean ± SD, as percentage changes or differences from basal values. The error bars in the figures indicate SD. Data were compared between groups at corresponding time points by Mann-Whitney *U* test (StatView, Institute Inc., Cary, NC). The level of confidence was placed at 95% for all experiments.

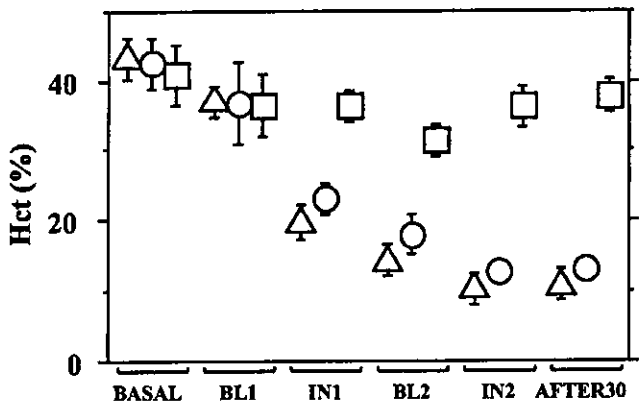

Figure 2. Changes in hematocrit in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group.

Results

Hemorrhagic Shock Resuscitation

Hemodynamic, blood gas, and intestinal measurements were performed in rabbits. After IN2, the hematocrit (Hct) (**Figure 2**) decreased from approximately 40% to 10% in both alb and HbValb groups. This indicated that approximately 40% of the circulating blood volume was actually replaced twice.

In **Table 2**, values at BASAL are shown for parameters representing hemodynamics, arterial blood gas, and intestinal perfusion. No significant intergroup differences were observed regarding these parameters. Therefore, the subsequent data changes are shown as percentage changes or differences from values at BASAL.

Mean arterial pressure (MAP) (**Figure 3a**) declined sharply after bleeding but rapidly recovered after infusion. There were no significant differences between groups. Heart rate (HR) (**Figure 3b**) tended to decrease slightly during the course of the experiment, but there were no significant differences between groups. Superior mesenteric aortic (SMA) blood flow (**Figure 3c**) declined sharply after bleeding but rapidly recovered after

Table 2. Basal Values of Measured Parameters

Basal Values	Alb Group	HbValb Group	RBCalb Group
Mean arterial pressure (mm Hg)	120 ± 13	110 ± 21	132 ± 19
Heart rate (beats/min)	233 ± 23	250 ± 33	240 ± 45
PaO_2 (Torr)	97.1 ± 11.0	92.3 ± 7.8	99.0 ± 9.7
$PaCO_2$ (Torr)	31.0 ± 4.7	30.1 ± 2.9	30.4 ± 2.6
Arterial base excess (mmol/L)	−3.2 ± 3.6	−2.9 ± 4.2	−4.8 ± 2.0
Superior mesenteric arterial flow (ml/min/kg)	22.0 ± 10.0	32.2 ± 11.2	36.9 ± 21.1
Intestinal mucosal pH	7.4 ± 0.1	7.4 ± 0.3	7.4 ± 0.1
Intestinal tissue PO_2 (Torr)	21.4 ± 3.4	18.4 ± 4.8	19.8 ± 6.0
Superior mesenteric venous PO_2 (Torr)	45.8 ± 3.1	42.2 ± 9.4	51.4 ± 8.9

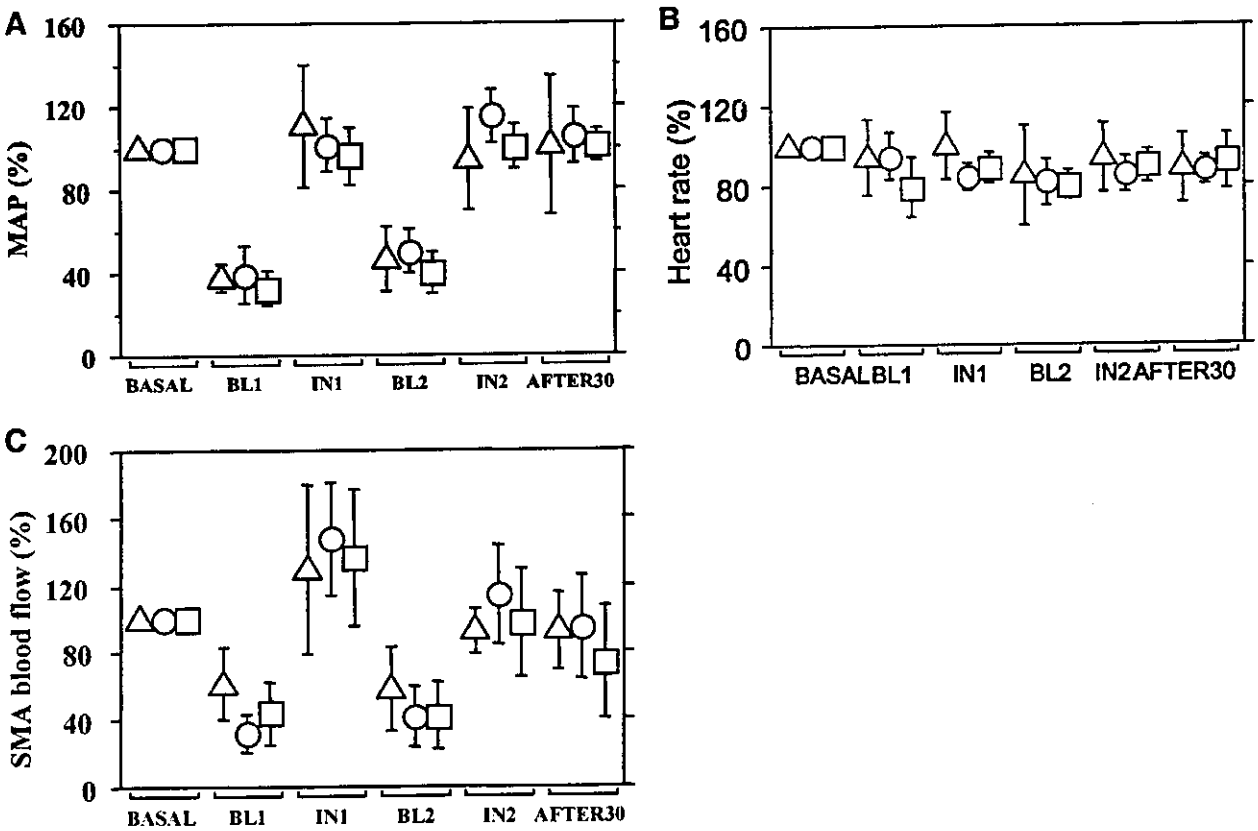

Figure 3. Changes in hemodynamic parameters from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group.

infusion. There were no significant differences between groups.

Arterial oxygen tension (PaO_2) (**Figure 4a**) tended to increase slightly during bleeding and infusion in all the groups. There were no significant differences between groups. Arterial carbon dioxide tension ($PaCO_2$) (**Figure 4b**) remained stable throughout the study in all the groups. Systemic base excess (BE) (**Figure 4c**) declined significantly in the alb group compared with the RBCalb group at BL2. At IN2 and AFTER30, BE in the alb group was significantly lower compared with both HbValb and RBCalb groups.

In the alb group, pHi (**Figure 5a**) declined significantly compared with both HbValb and RBCalb groups beyond BL2. Intestinal tissue oxygen tension (**Figure 5b**) declined after bleeding but recovered to baseline by infusion in the HbValb and RBCalb groups but not in the alb group. The differences were significant beyond IN2. Superior mesenteric venous (SMV) oxygen tension (**Figure 5c**) declined sharply after bleeding but rapidly recovered close to baseline after infusion in all the groups. However, at AFTER30, it significantly increased in the alb group compared with both HbValb and RBCalb groups.

Histologic Examination

No significant abnormalities or differences among groups were observed in any of the organs examined in the rabbits.

Plasma Level of Tumor Necrosis Factor-α

In the rats, TNF-α concentration in plasma (pg/ml) was increased approximately 40-fold in the alb group (4,634 ± 4,276) compared with the HbValb group (124 ± 65). In the RBCalb group, it was below detection limit (<25).

Discussion

Peripheral tissue perfusion is controlled in response to changes in systemic hemodynamics. Intestinal perfusion is known to be one of the first to decline in hemorrhagic shock when the redistribution of systemic blood flow occurs to other vital organs such as the heart and the brain. However, it is also known that the loss of adequate intestinal function caused by insufficient perfusion leads to serious complications such as bacterial translocation and cytokine production,[17] which can eventually lead to mortality even when other vital organs are initially well sustained. It has been shown that indices such as intestinal mucosal pH are valid in assessing the severity of shock, as well as predicting prognosis.[18] To this end, in this study, we observed parameters of intestinal perfusion in addition to systemic hemodynamic parameters to evaluate the applicability of HbV in hemorrhagic shock resuscitation.

In the present study, in the rabbits, shock resuscitation with albumin satisfactorily restored parameters such as MAP and HR. Lung function was also maintained as shown by the

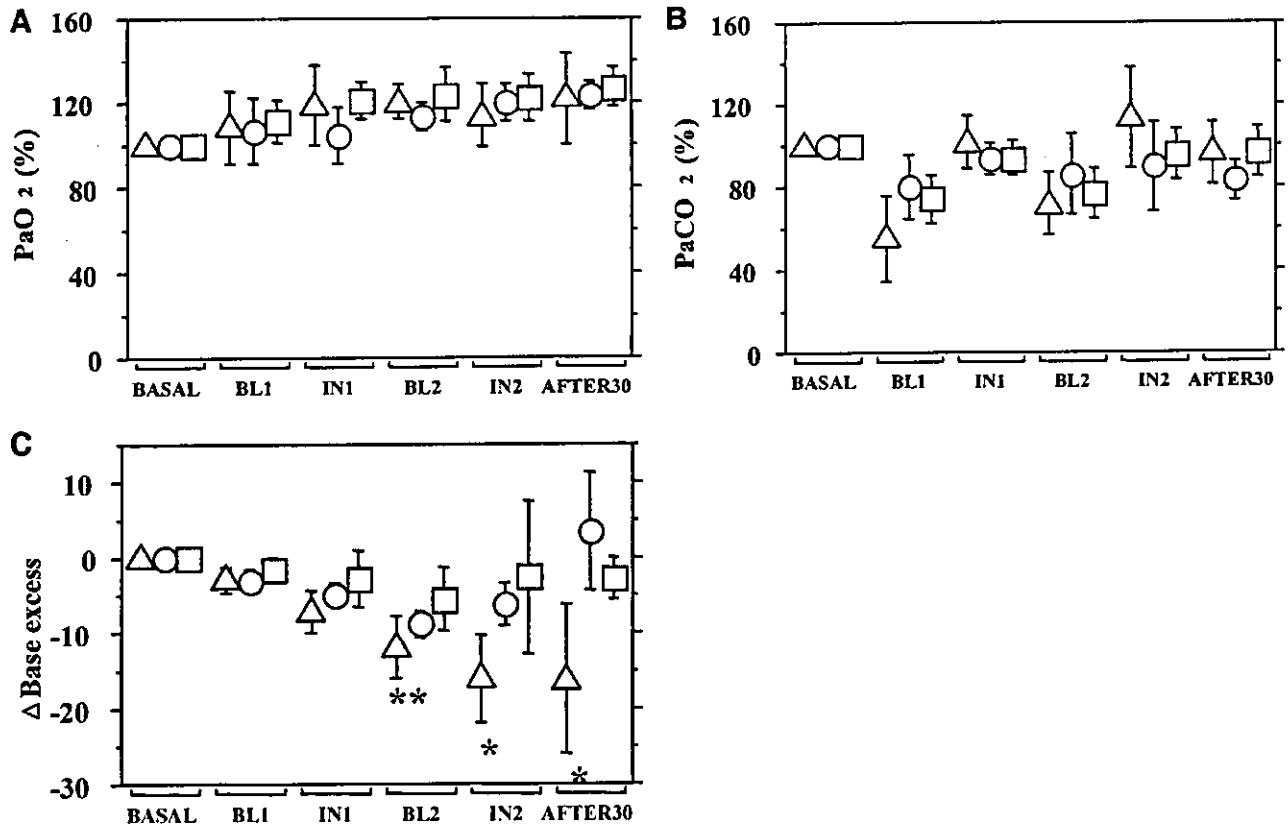

Figure 4. Changes in arterial blood gas parameters from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group. $^{*}p < 0.05$ *vs.* HbValb and RBCalb groups; $^{**}p < 0.05$ *vs.* RBCalb group.

systemic oxygen and carbon dioxide tension. However, systemic base excess significantly declined in the alb group indicating peripheral hypoperfusion, and our data show that one such organ is the intestine. Even though SMA blood flow was maintained, lack of peripheral perfusion in the alb group was depicted by the significant decline in pHi and intestinal tissue oxygen tension. The subsequent significant increase in SMV oxygen tension was most likely brought about by the shutdown of peripheral circulation leading to shunting of SMA blood. We consider that all of these changes resulted from the impairment in cardiac function caused by decreased oxygen content in the alb group, which subsequently limited the oxygen delivery to the cardiac muscles. It is likely that a longer observation period was required for these changes to become morphologically apparent on histology. However, most of the animals in the alb group could not survive beyond 30 minutes after the second infusion in this study design.

TNF-α is believed to be an important mediator of SIRS. It has been reported that the intestine is a major source of TNF-α production during hemorrhagic shock.[19] We used rats for the measurement of plasma TNF-α. Ideally, the assay for TNF-α should have been performed in rabbits. However, we were not able to find an appropriate TNF-α antibody to perform ELISA in rabbits. On the other hand, we had previously performed TNF-α assay in the rat, and the assay technique was well established. In our preliminary shock resuscitation experiments in rats, we found that withdrawal of 40% of estimated circulating blood volume reduced MAP to approximately 40% of baseline (data not shown). Also, we have previously reported that withdrawal of 50% of estimated circulating blood volume in rats reduced MAP to approximately 20% of baseline, and base excess declined from 0 to approximately −6.[9] From these data, we extrapolated that the hemodynamic changes would be similar in rats compared with rabbits under the same shock resuscitation protocol. Therefore, we decided to perform the TNF-α measurements in rats. Under the same experimental protocol, we saw a significant increase in the plasma levels of TNF-α in the alb group. This was effectively suppressed in the HbValb group, although not quite to the level of RBCalb group. In this particular experiment, there is no evidence to show that the hemodynamic changes or the changes in the intestinal parameters were the same in the rats compared with the rabbits. However, we believe that the substantial intergroup differences in TNF-α in the rats, although not directly, provide support that intestinal, and possibly other organ damage, was reduced by shock resuscitation with HbV.

These data show that significant covert damage to the intestine is present in the alb group despite seemingly adequate systemic hemodynamics. This was because of the deficiency of blood oxygen content despite sufficient volume. In contrast, systemic, as well as intestinal, perfusion in the HbValb group were well sustained and were comparable with the RBCalb group. Plasma TNF-α level was also effectively reduced in the

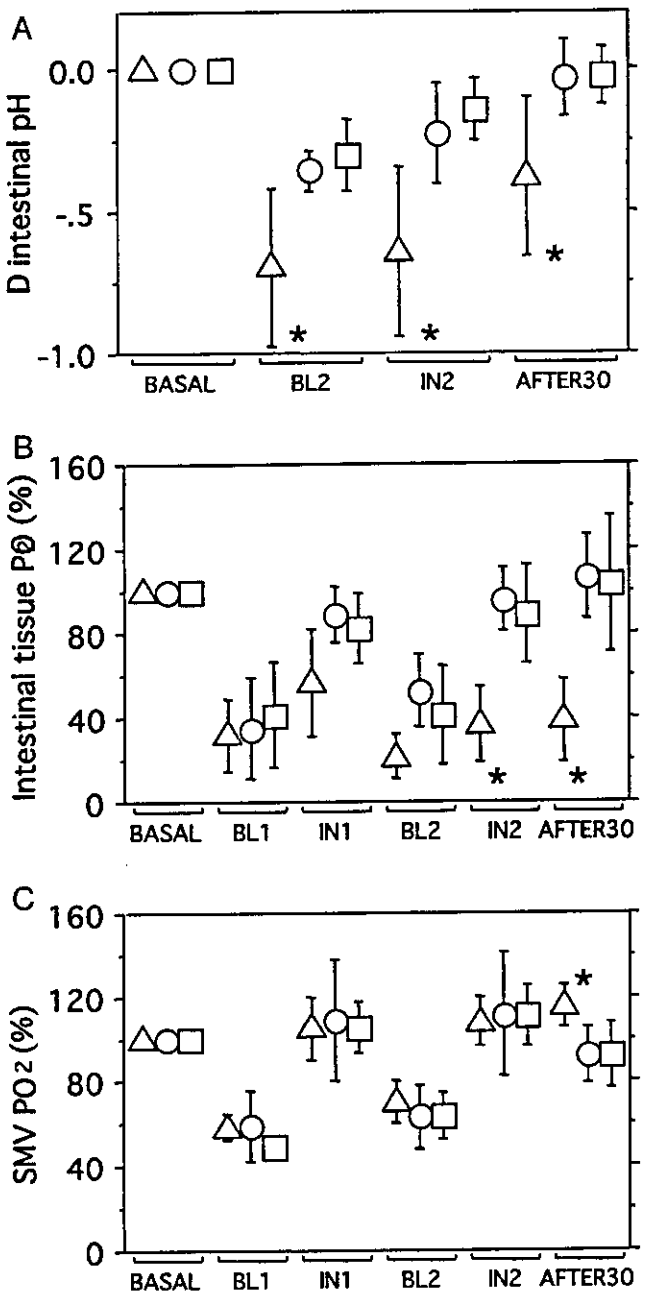

Figure 5. Parameters representing intestinal perfusion are shown as percentage changes or difference from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group. *$p < 0.05$ *vs.* HbValb and RBCalb groups.

HbValb group, close to the RBCalb group. These data collectively indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. One of the powerful advantages of HbV is that its properties, such as oxygen binding and release, viscosity, and colloid osmotic pressure, can be manipulated by changing the amount of allosteric effector in HbV and the plasma expander in which to suspend HbV. We believe that currently ongoing optimization of these properties will further improve the efficacy of HbV in shock resuscitation.

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

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ABSTRACT

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

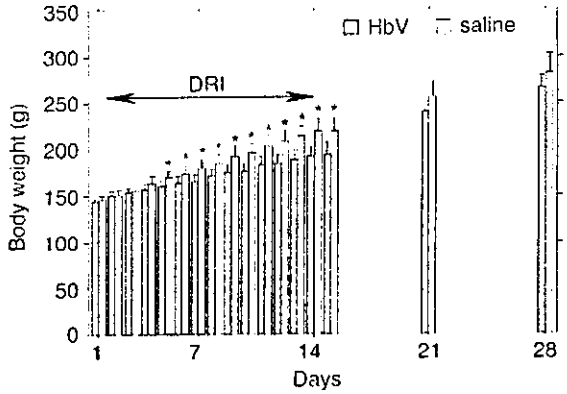

Fig. 1. Time course of the gain in body weight during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. Both groups showed monotonous increases; however, after the 5th day, a significant difference was observed. Seven days after the final infusion, there were no significant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

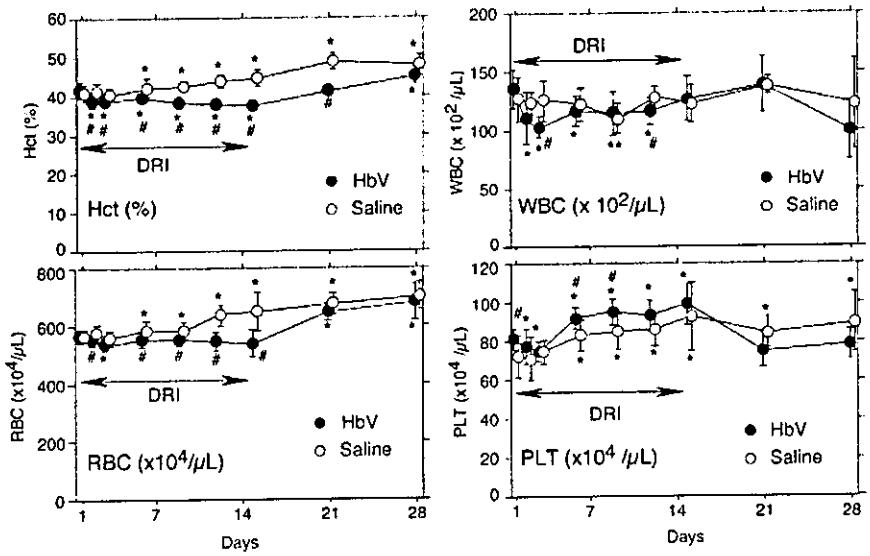

Fig. 2. Time course of Hct and blood cell counts during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. #, significantly different between the groups ($p < 0.05$). *, significantly different versus the baseline values ($p < 0.05$).

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

Coagulation Test. A coagulation test indicated that the HbV group showed a slight prolongation in APTT from 24.3 ± 1.3 s at the baseline to 36.0 ± 11.8 s 1 day after the DRI but changed to 40.3 ± 20.3 s after 14 days with marked individual variations. On the other hand, there was no noticeable change in the PT for the HbV group (Fig. 4). The fibrinogen concentration (baseline, 223 ± 12 mg/dl) significantly increased for all groups (HbV group, 1 day after DRI, 289 ± 22 mg/dl; 14 days after DRI, 255 ± 11 mg/dl), probably due to the stress of infusion and influence on the liver function.

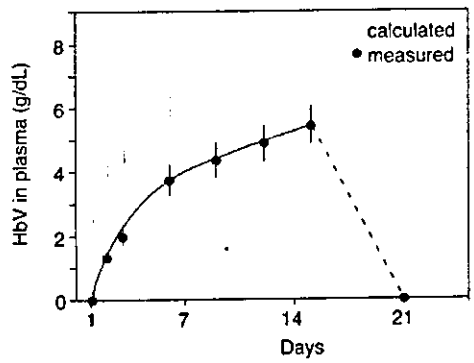

Fig. 3. Time course of the HbV concentration in the plasma phase during and after DRI of HbV for 14 days at a dose rate of 10 ml/kg/day. It is assumed that immediately after the first infusion, the concentration should be around 3 g/dl, and 1 day later, it decreased to about 1.2 g/dl. Immediately after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

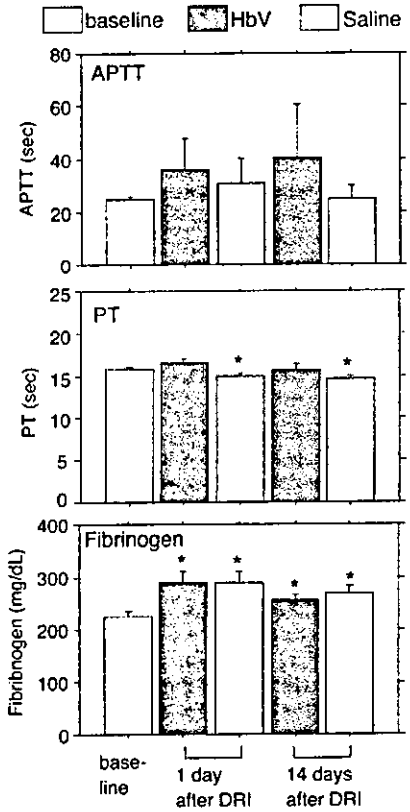

Fig. 4. Parameters for blood coagulation, APTT and PT, and fibrinogen concentration after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

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

TABLE 1

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

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

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

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

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

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

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

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

TABLE 2

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

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

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

[a] $n = 5$.

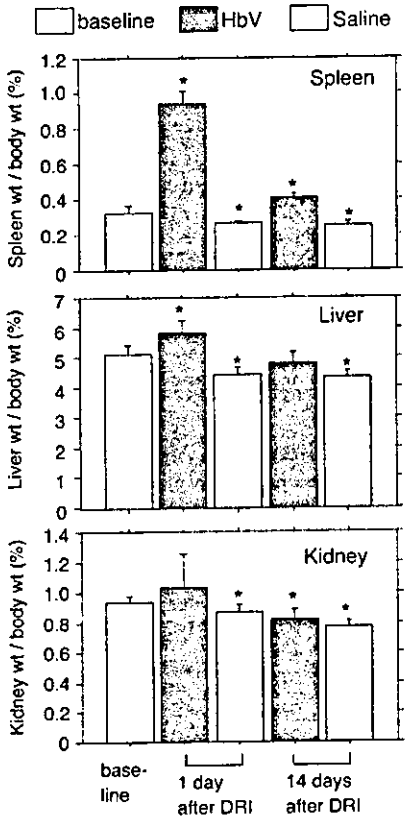

Fig. 5. Changes in organ weights (spleen, liver, and kidney) after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

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

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

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

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

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

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

Discussion

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

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

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