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Hemoglobin-Vesicles as Oxygen Carriers

Influence on Phagocytic Activity and Histopathological Changes in Reticuloendothelial System

Hiromi Sakai,* Hirohisa Horinouchi,† Kenichi Tomiyama,* Eiji Ikeda,‡ Shinji Takeoka,* Koichi Kobayashi,† and Eishun Tsuchida*

From the Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo; and the Departments of Surgery† and Pathology,‡ Keio University School of Medicine, Tokyo, Japan*

Hemoglobin-vesicles (HbV) have been developed for use as artificial oxygen carriers (particle diameter, 250 nm) in which a purified Hb solution is encapsulated with a phospholipid bilayer membrane. The influence of HbV on the reticuloendothelial system was studied by carbon clearance measurements and histopathological examination. The HbV suspension ([Hb] = 10 g/dl) was intravenously infused in male Wistar rats at dose rates of 10 and 20 ml/kg, and the phagocytic activity was measured by monitoring the rate of carbon clearance at 8 hours and at 1, 3, 7, and 14 days after infusion. The phagocytic activity transiently decreased one day after infusion by about 40%, but it recovered and was enhanced at 3 days, showing a maximum of about twice the quiescent level at 7 days, and then returned to the normal value at 14 days. The initial transient decreased activity indicates a partly, but not completely, suppressed defensive function of the body. The succeeding increased phagocytic activity corresponds to the increased metabolism of HbV. The histopathological examination with anti-human Hb antibody, hematoxylin/eosin, and oil red O stainings showed that HbV was metabolized within 7 days. Hemosiderin was very slightly confirmed with Berlin blue staining at 3 and 7 days in liver and spleen, though they completely disappeared at 14 days, indicating that the heme metabolism, excretion or recycling of iron proceeded smoothly and iron deposition was minimal. Electron microscopic examination of the spleen and liver tissues clearly demonstrated the particles of HbV with a diameter of about 1/40 of red blood cells in capillaries, and in phagosomes as entrapped in the spleen macrophages and Kupffer cells one day after infusion. The vesicular structure could not be observed at 7 days. Even though the infusion of HbV modified the phagocytic activity for 2 weeks, it does not seem to cause any irreversible damage to the phagocytic organs. These results offer important information for evaluating the safety issues of HbV for clinical use. ***(Am J Pathol 2001, 159:1079–1088)***

Phospholipid vesicles encapsulating concentrated human hemoglobin (Hb, Hb vesicles, HbV) can serve as an oxygen carrier with sufficient oxygen transporting ability comparable to blood.[1–5] The advantages of Hb-based oxygen carriers are the absence of a blood type antigen and infectious virus, a small particle size for the penetration through constricted vessels where red blood cells cannot penetrate, and stability for long-term storage.[6] These characteristics will make it possible to use the oxygen carriers both in elective and emergency situations without blood type matching and virus tests. In this sense, infusion of oxygen carriers becomes superior to the conventional blood transfusion which still has the potential of mismatching, infection such as HIV and hepatitis virus, and graft-versus-host disease, and the problems of a short period of preservation. Even though the acellular Hb modifications, including polymerized Hb and polymer-conjugated Hb, are now undergoing the final stages of clinical trials,[7–10] the cellular structure of HbV (particle diameter, ~ 250 nm) most closely mimics the characteristics of natural red blood cells such as the cell membrane function of physically preventing direct contact of Hb with the components of blood and vasculature during circulation. In comparison with some acellular Hb modifications, Hb encapsulation in vesicles not only prolongs the circulation time, but also 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.[11–13]

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Address reprint requests to Eishun Tsuchida, Professor, Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University, Tokyo 169–8555, Japan. E-mail: eishun@mn.waseda.ac.jp.

Free Hb released from RBC is rapidly bound to haptoglobin and removed from the circulation by hepatocytes. However, when the Hb concentration exceeds the haptoglobin binding capacity, unbound Hb is filtered through the kidney where it is actively absorbed. When the reabsorption capacity of the kidney is exceeded, hemoglobinuria and eventually renal failure occur.[14] The encapsulation of Hb completely suppresses renal excretion, though HbV particles as well as phospholipid vesicles (liposomes) or oil emulsions in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS).[15–17] A chemically modified (pyridoxalated) Hb was also reported to be captured by RES.[18] The main function of RES is protective elimination of foreign materials such as viruses, bacteria, metastatic tumor cells, and bacterial endotoxins.[19] It has been anticipated that the accumulation of phospholipid vesicles in phagocytic cells may lead to impairment or even blockade of their function.[20] This may cause adverse effects such as decreased resistance to infection, the metastatic spread of tumors, or spillover of endotoxins from the gut. Clinically approved Fluosol-DA (Green Cross Co., Osaka, Japan) as an oxygen carrier and lipid emulsion as a nutrient are trapped by the phagocytic cells.[21–24] Several reports measured the phagocytic activity by the carbon clearance method and clarified that the phagocytic activity is transiently depressed after the infusion and then recovered and enhanced.[25–28]

The advantages of our HbV over the conventional Hb vesicles[29–31] are a high encapsulation efficiency of Hb in phospholipid vesicles thus the total amount of lipids is significantly reduced,[32–33] the surface modification of HbV with poly(ethylene glycol) that allows better hemodynamics due to the suppression of intervesicular aggregation and reduced viscosity,[34–36] reduced complement activation due to the suppression of interaction with plasma proteins,[37] and longer circulation time and a moderate rate of entrapment and metabolism.[38] We thought these revised characteristics may be effective to maintain microcirculation and to reduce the burden on RES. In this study, the effect of HbV infusion on the RES function was analyzed by the carbon clearance measurement,[25,28,39] and also its metabolism and the influence on the tissue parenchymal cells was confirmed by histopathological examination.

Materials and Methods

Preparation of poly(ethylene glycol)-modified Hb-vesicles (HbV) polyethylene glycol (PEG)-modified HbV was performed at Waseda University under sterile conditions as previously reported in the literature.[12,32,35] Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan). The encapsulated carbonylhemoglobin (HbCO, 38 g/dl) contained 5.9 mmol/L of pyridoxal 5′-phosphate (PLP, Merck, Whitehouse Station, NJ) as an allosteric effector at a molar ratio of Hb/PLP = 3, and 5 mmol/L of homocysteine (Aldrich, Milwaukee, WI) as a reductant. The lipid bilayer was composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol at a molar ratio of 5/5/1 (Nippon Fine Chemicals, Osaka, Japan)]. The HbCO solution and the lipids were mixed and stirred for 12 hours at 4°C. The resulting mutilamellar vesicles were extruded through membrane filters using Remolino™ (Millipore, Bedford, MA) with a final filter pore size of 0.22 μm. After rinsing with saline, the HbV surface was modified with PEG (molecular weight 5 kd, 0.3 mol % to the outer surface of lipids) using 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (Sunbright DSPE-50H, H salt type, NOF Co., Tokyo, Japan), where succinic acid is a crosslinker between PEG and DSPE. The hydrophobic alkyl chains of PEG-DSPE are inserted into the lipid bilayer of HbV by mixing the HbV suspension with a saline suspension at 37°C for 2 hours. After decarbonylation of HbCO to HbO_2, the resulting PEG-modified HbV was ultracentrifuged to remove the unintroduced PEG-lipid, and redispersed in saline at the Hb concentration of 10 g/dl. The suspension was then filtered through sterilizable filters (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], <5%; [HbCO], < 3%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 32 Torr.

Injection of Sample Suspensions

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).

Experiments were carried out using 70 male Wistar rats (200–210 g, Charles River Co., Tokyo, Japan). They were anesthetized with diethylether, and the sample suspension was infused into the tail vein. The sample was either HbV (10 ml/kg, n = 15; 20 ml/kg, n = 19) or saline (20 ml/kg, n = 15) and 10 wt% of Intralipid™ suspension (Pharmacia, Stockholm, Sweden) 20 ml/kg, n = 15). Six animals were used to obtain the control values. All of the rats were housed in cages and provided with food and water *ad libitum* in a temperature controlled room on a 12 hour dark/light cycle.

Carbon Clearance Measurement

After 8 hours and 1, 3, 7, and 14 days, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (~ 100 mg/kg body weight, Abbott Lab., North Chicago, IL). Polyethylene tubes (PE-50, Natsume Co., Tokyo) were implanted in the jugular vein. A carbon particle solution (Fount India Ink, Pelikan Co., Hannover, Germany) was diluted to 16 mg/ml with saline and infused at 10 ml/kg within 1 minute. The pink-colored rat skin immediately turned to black, indicating that the carbon particles were circulating throughout the body. Four, 10, and 20 minutes later, about 120 μl of blood was

withdrawn from the vein, and exactly 50 μl of blood was diluted with 5 ml of a 0.1% sodium bicarbonate solution in a cuvette for spectrophotometer. Absorption at 675 nm was measured with the spectrophotometer (UV-2000, Shimadzu Co., Tokyo, Japan). The control blood was also measured before infusing the carbon particle solution. The phagocyte index (K) was calculated with the equation: $K = 1/(t_2-t_1) \times \ln(C_1/C_2)$ where C_1 and C_2 are the concentrations (absorbance) at time t_1 and t_2 (minutes), respectively. After the experiment, the animals were laparotomized to be sacrificed with acute bleeding from the abdominal aorta and to obtain the liver, spleen, and kidney, and then the lung and heart were resected *en bloc* for a histopathological study. The organs were soaked in 10% formalin immediately after the resection.

Histopathological Study

Paraffin sections were prepared from the 10% formalin-fixed organs, and stained with hematoxylin/eosin, anti-human Hb antibody, Berlin blue, and oil red O stainings. The human Hb in the HbV particles in the tissue was confirmed by staining with a rabbit polyclonal antibody against human Hb (DAKO A/S, Copenhagen, Denmark) as the primary antibody. This antibody does not cross-react with rat hemoglobin (which was evident from the result that rat red blood cells were not stained). Reaction with the second antibody and color development were performed with the Ventana alkaline phosphatase RED detection kit using the Ventana NX system (Ventana Med. System, Inc., Tucson, AZ). The percentage of the stained area was calculated with a computer software (IPLab, Fairfax, VA). The presence and location of hemosiderin including free irons released by the metabolism of heme were confirmed by Berlin blue stain. The neutral lipid deposition, which might be generated during the metabolism of the phospholipid components of the bilayer membrane of HbV, were examined by oil red O staining of the sliced organ specimens directly prepared from the formalin-fixed organs.

To visualize the morphological changes in the HbV particles in the spleen and liver, transmission electron microscopic observation (with a high magnification) was performed. The spleen and liver, taken from the rat without carbon particle infusion, were cut in about 2 mm^3 portions in 2.5% glutaraldehyde solution and then stored in 8% sucrose solution (0.1 mol/L phosphate buffer, pH 7.4). The fixed organs were then washed with 0.1 mol/L phosphate buffer, and stained with 2% osumic acid solution at 4°C for 2 hours. Next, the organs were first dehydrated with ethanol solution by stepwise increases in the ethanol content (50, 60, 70, 80, 90, 95, and 100%) for 10 minutes during each step, washed with propylene oxide, and then polymerized using Quetol 812 at 60°C for 28 hours. The obtained samples were sliced into 60 to70-nm sections by using an Ultracut S microtome. The sliced samples were stained with 3% uranyl acetate solution for 16 to 20 minutes and then treated with Satoh's lead solution (lead acetate, lead nitrate, and lead citrate) in citrate for 5 minutes, washed, and dried. The sample was observed and a picture taken with a transmission electron microscope (TEM, JEM-100CX, JEOL, Tokyo, Japan).

Blood Serum Clinical Chemistry

The rats receiving HbV but not carbon particles were used to analyze blood serum clinical chemistry ($n = 15$). After 8 hours and 1, 3, 7 and 14 days, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. Polyethylene tubes (PE-50) were implanted in the carotid artery and about 4 ml of blood was withdrawn in heparinized syringe. Since HbV particles interfere with some analytes of serum clinical chemistry, the blood was ultracentrifuged (50,000 $\times$ g for 20 minutes) to completely remove the HbV particles in advance. The serum samples were stored in a refrigerator (−80°C) until the analyses. To evaluate function of liver as one of the main organs for the HbV metabolism, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were selected as the analytes (BML Inc., Kawagoe, Japan).

Data Analysis

Differences between the treatment groups were analyzed using a one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) test. A paired t-test was used to compare the time dependent changes within each group. The changes were considered statistically significant if $P < 0.05$.

Results

Body and Spleen Weights

All of the rats tolerated the overdose of the sample solutions. The original body weights were 210 to 225 g on average (Figure 1a). The body weight decreased, especially in the 20 ml/kg HbV group, by about 10% one day after the HbV administration. However, the body weight of the HbV groups returned to that of the control groups within 3 days and they grew normally.

The two HbV groups showed splenomegaly (Figure 1b). The spleen weight increased for the 20 ml/kg HbV group by about 500 mg, which is about 70% of the infused amount of HbV (3300 mg/kg $\times$ 0.21 kg = 693 mg), and the spleen weight tended to remain even after 2 weeks. The 10 ml/kg HbV group showed a spleen weight increase at 1 week and tended to decrease at 2 weeks.

Changes in Phagocytic Activity

The phagocytic index (K) dropped at 8 hours or 1 day after the HbV infusion by about 30 to 50%, though it never completely saturated (Figure 2). In the case of the 10 ml/kg HbV group, the K value recovered 3 days later and showed a maximum value at 1 week, and then ceased at 2 weeks. For the 20 ml/kg HbV infusion, the significantly

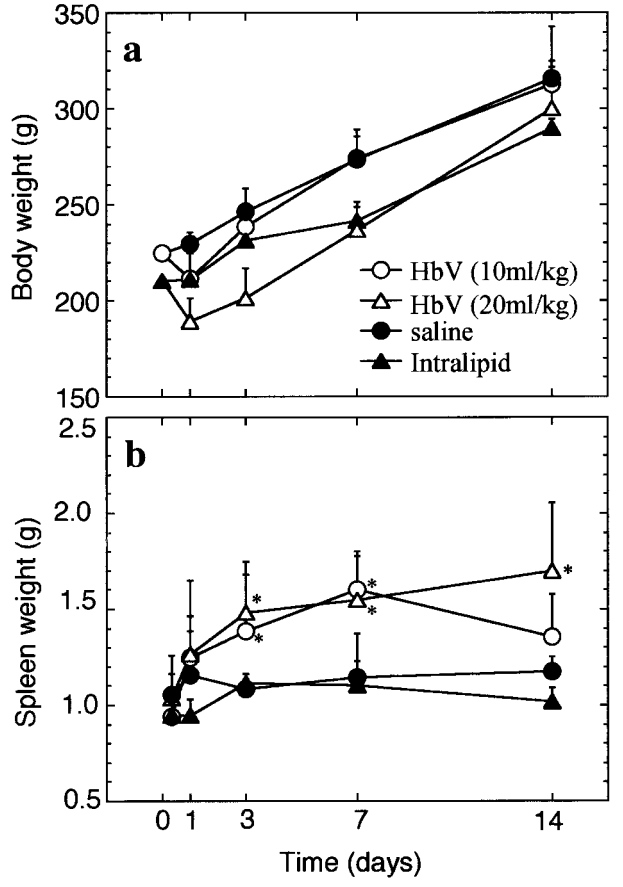

Figure 1. Changes in body weight (**a**) and spleen weight (**b**) after infusion of HbV (10 and 20 ml/kg), saline and Intralipid as controls. The values are mean ± SD. *Significantly different from the saline group ($P < 0.05$).

high value of *K,* about twice the baseline value, was observed at 1 week, and then it ceased at 2 weeks. Thus the dramatic changes in the phagocytic activities were not irreversible. The changes in *K* for the saline and lipid microsphere groups were minimal.

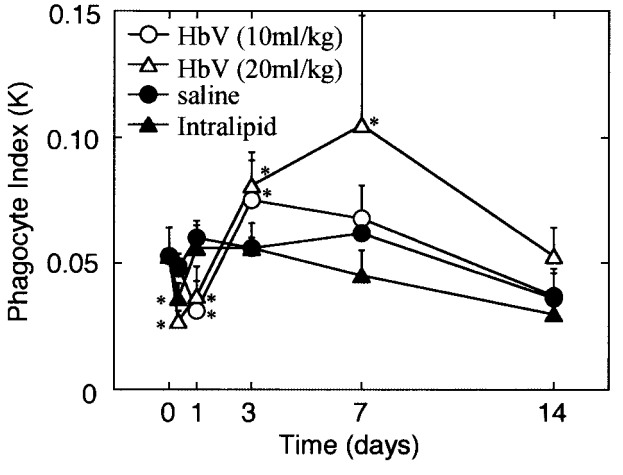

Figure 2. Changes in phagocytic activity after infusion of HbV (10 and 20 ml/kg), saline and Intralipid as controls. The values are mean ± SD. The phagocytic activity was obtained with the carbon clearance measurement. *Significantly different from the basal value, $K = 0.053 \pm 0.011$ ($P < 0.05$).

Histopathological Examination by Staining

The histopathological examination of the spleen and liver after the 20 ml/kg infusion is shown in Figure 3. The human Hb in HbV particles were stained as red-colored portions with anti-human Hb antibody as a primary antibody. It was confirmed in advance with smears of human and rat blood and HbV suspension that the antibody reacts with only human Hb but not with rat Hb.

The spleen and liver accumulated the HbV particles in the macrophages and the Kupffer cells (Figure 3). A significant amount of macrophages in spleen entrapping HbV particles was seen in the red pulp zone as red-colored domains (Figure 3, a, c, and e). The total area of the red-colored portion in the red pulp zone was 31.0 ± 6.1% at one day, then it gradually decreased to 5.1 ± 2.0% after 3 days, and to less than 0.05% after 7 days (Figure 4). On the other hand, a significant amount of carbon particles was seen in the marginal zone around the white pulp, where lymph cells are located, indicating the enhanced phagocytic activity (Figure 3, a, c, and e). For the liver, Kupffer cells trapping HbV particles were seen as a red-stained area at one day after the infusion (Figure 3b). After 3 days, the HbV as well as a large amount of carbons were seen in the same position (Figure 3d). The HbV particles completely disappeared 7 days after infusion while the Kupffer cells trapping a large amount of carbon were observed which corresponded to the enhanced phagocytosis (Figure 3f). The total area of the red-colored portion was 7.6 ± 1.9% at one day, then it gradually decreased to 1.3 ± 0.2% after 3 days, and almost completely disappeared after 7 days (Figure 4).

There was a very slight signal with Berlin blue stain in the macrophages and the white pulp zone in the spleen and and in the Kupffer cells and Glisson's sheath in the liver at 3 and 7 days (Figure 5, a and b). However, at 14 days, no stain was confirmed either in the spleen or liver, indicating that the heme metabolism from Hb proceeded smoothly.

The oil red O staining on all of the organs after 20 ml/kg of HbV infusion revealed that slight stains were confirmed only in liver 3 days after infusion (Figure 6). The dye, oil red O, locates in the domain of neutral lipid (e.g., triglyceride). Therefore, this result indicated that the metabolism of phospholipid components proceeded smoothly, and there was no deposition of the metabolites. In kidney and lung no significant pathological infarcts, such as capillary emboli with HbV particles, are noted in either organ (Figure 7, a and b).

Histopathological Examination by Transmission Electron Microscopy

Transmission electron microscopy (TEM) observations of the phagocytic cells clearly demonstrated the presence of HbV particles in the phagosomes and capillaries both in the liver and spleen after 1 day (Figure 8, a and b). The HbV diameter is about 250 nm which is about 1/40 of red blood cells. Since one Hb contains four irons and a resulting higher electron density, the Hb-vesicles as well

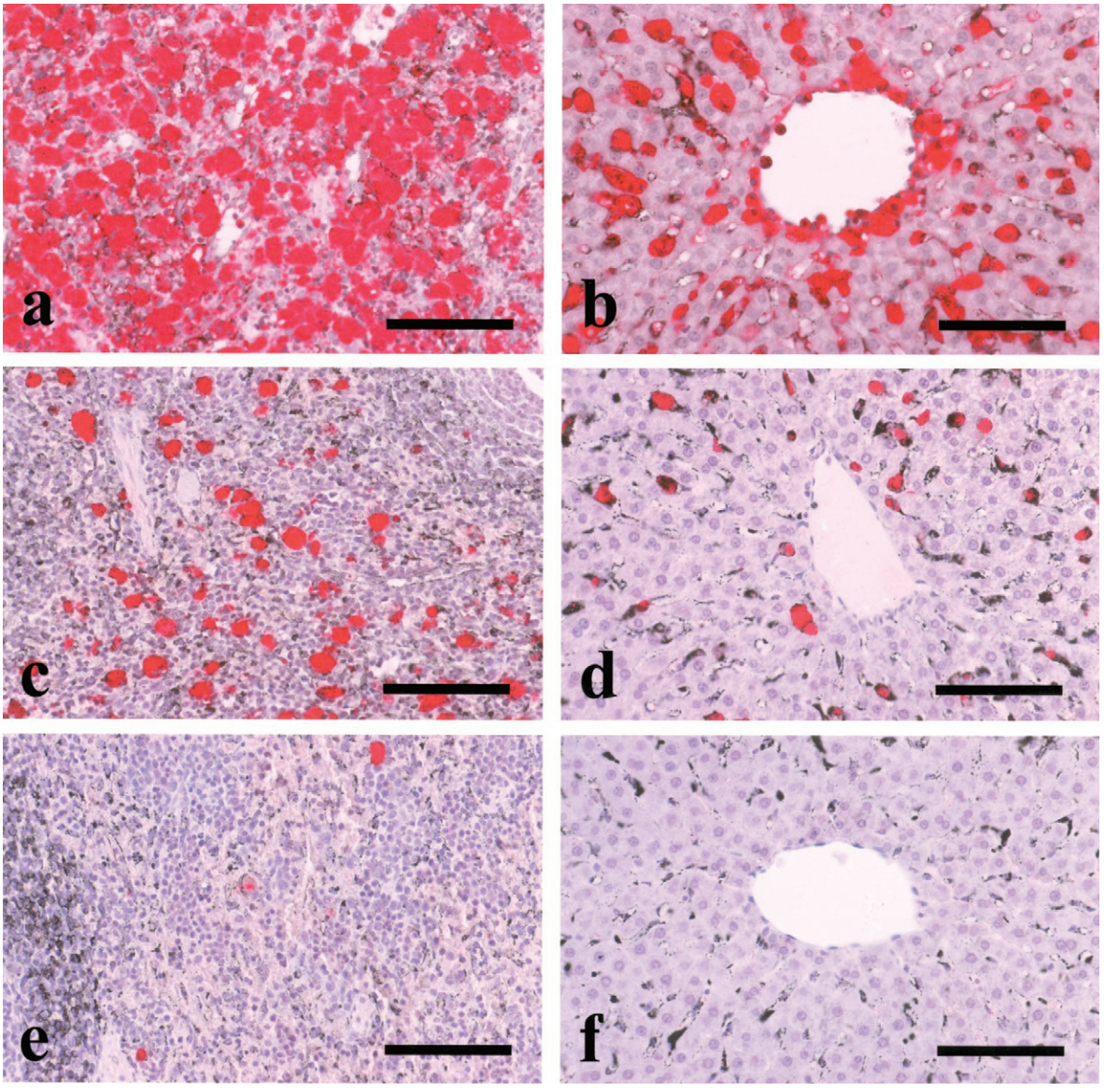

Figure 3. Human Hb in the rat spleens (**a, c, e**) and livers (**b, d, f**) 1 (**a, b**), 3 (**c, d**), and 7 (**e, f**) days after the bolus infusion of 20 ml/kg HbV suspension. The tissues were stained with rabbit polyclonal antibody against human Hb to examine the accumulation of human Hb from HbV. Red-stained signals correspond to human Hb, and black signals to injected carbon particles. Accumulation of human Hb is observed in both spleens and livers 1 and 3 days after infusion (**a, b, c, d**), while no significant accumulation is seen in both organs after 7 days (**e, f**). Human Hbs are located in macrophages in spleen and in Kupffer cells in liver. Scale bars, 100 μm.

as red blood cells are black in color. Some HbV are seen near the RBCs indicating that they are in capillaries and most of the HbV are captured by the phagocytic cells. After 3 days, HbV cannot be seen in the capillaries and the HbV is mostly captured by the RES. After 7 days, no phagosomes with HbV particles can be observed (Figure 8, c and d).

Blood Serum Clinical Chemistry

The AST and ALT values before the infusion of HbV were 65.4 ± 2.7 units/l and 29. 6 ± 3.5 units/l, respectively. After the bolus infusion of HbV at 20 ml/kg dose rate, they significantly increased to 483 ± 85 units/l and 149 ± 5 units/l at 8 hours, respectively. However, after 3 days they returned to the original levels; AST was 47.5 ± 7.8 units/l and ALT was 44.0 ± 15.5 units/l. The values were almost unchanged until 14 days after the infusion.

Discussion

Our previous studies demonstrated the sufficient oxygen transporting capability of the HbV suspension in animal tests which was comparable with red blood cells.[4,5] The clinical indications for the use of the HbV suspension as an artificial oxygen carrying fluid are estimated to be mainly preoperative or perioperative hemodilution, or resuscitation from hemorrhagic shock in emergency situations, both of which result in exchanging more than 20%

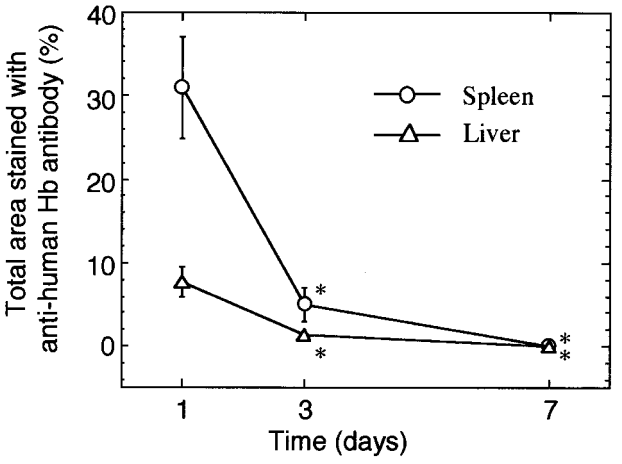

Figure 4. Changes in the total stained area (Figure 3) in the red pulp zone of spleen and in the liver after after the bolus infusion of 20 ml/kg HbV suspension. The values are mean ± SD of randomly selected five areas. *Significantly different from the value at 1 day ($P < 0.005$).

of original blood with the HbV suspension. Thus the dose amount is much greater than the infusion of stealth liposomes which are clinically used as vehicles for anti-tumor drugs[40,41] and DNA transfection.[42] After circulation in the blood stream, HbV particles are finally captured by RES in the same manner as the conventional phospholipid vesicles.[43,44] Therefore, the influence of HbV on the RES

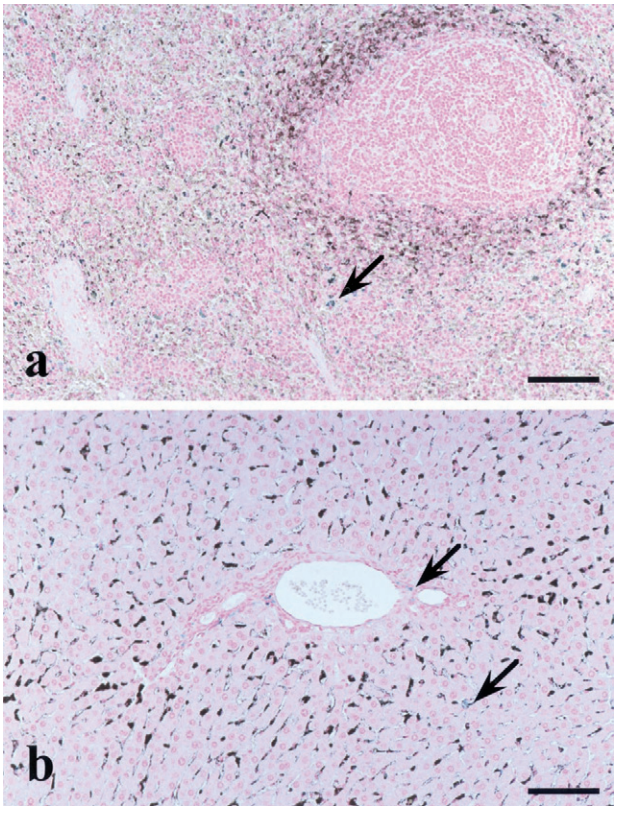

Figure 5. Hemosiderin in spleen (**a**) and liver (**b**) 7 days after the bolus infusion of 20 ml/kg HbV suspension. Berlin blue stain was performed to examine the presence of hemosiderin. Slight deposition of hemosiderin is observed in both spleen (**arrows** in **a**) and liver (**arrows** in **b**). Scale bars, 100 μm. Berlin blue stain.

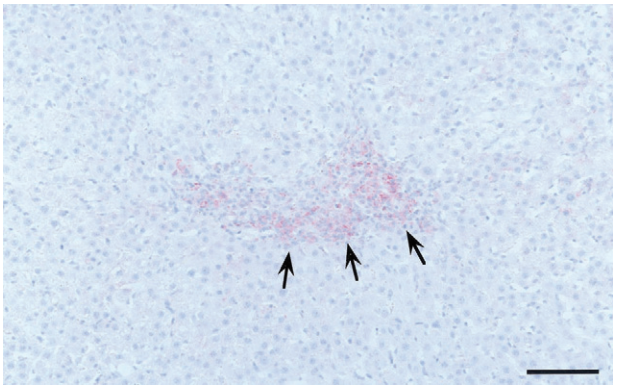

Figure 6. Lipid deposition in the liver 3 days after the bolus infusion of 20 ml/kg HbV suspension. The liver was stained with oil red O to confirm the presence of a domain of neutral lipid deposition. Slight lipid deposition is seen (**arrows**). Bar, 100 μm.

is one issue of safety evaluation that needs to be clarified to allow the use of HbV in clinical applications.

The carbon clearance measurement showed that the systemic phagocytic activity decreased by 40% soon after the HbV infusion. Since the circulation time of HbV at the used condition is about 20 to 30 hours, the peak of the HbV accumulation is estimated to be around 1 to 2 days after the infusion, and this corresponds to the significantly decreased phagocytic activity at 8 hours and 1 day. However, it recovered 3 days after the infusion, and then it increased by 200%. The same tendency of suppression and the succeeding enhancement of phagocytic activity

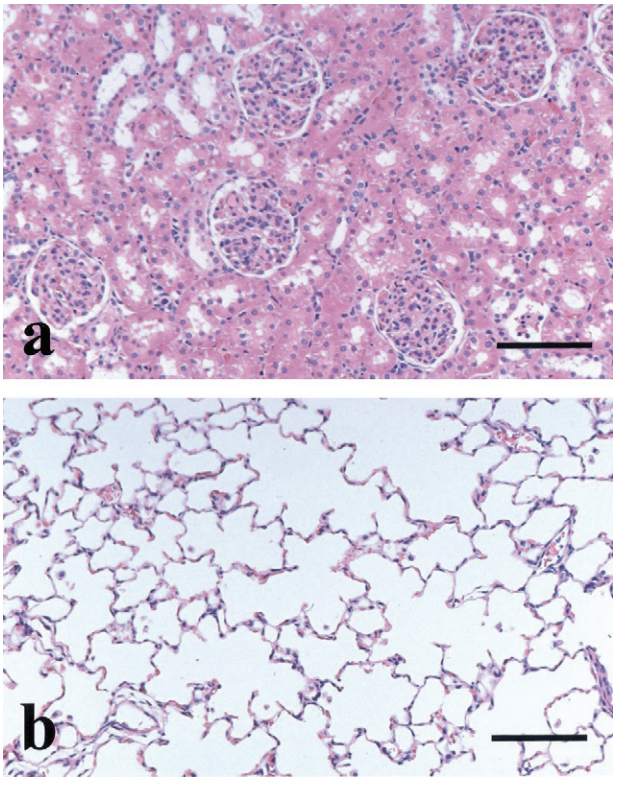

Figure 7. Histology of kidney (**a**) and lung (**b**) 1 day after the bolus infusion of 20 ml/kg HbV suspension. No significant pathological changes are noted in both organs. Bars, 100 μm. HE stain.

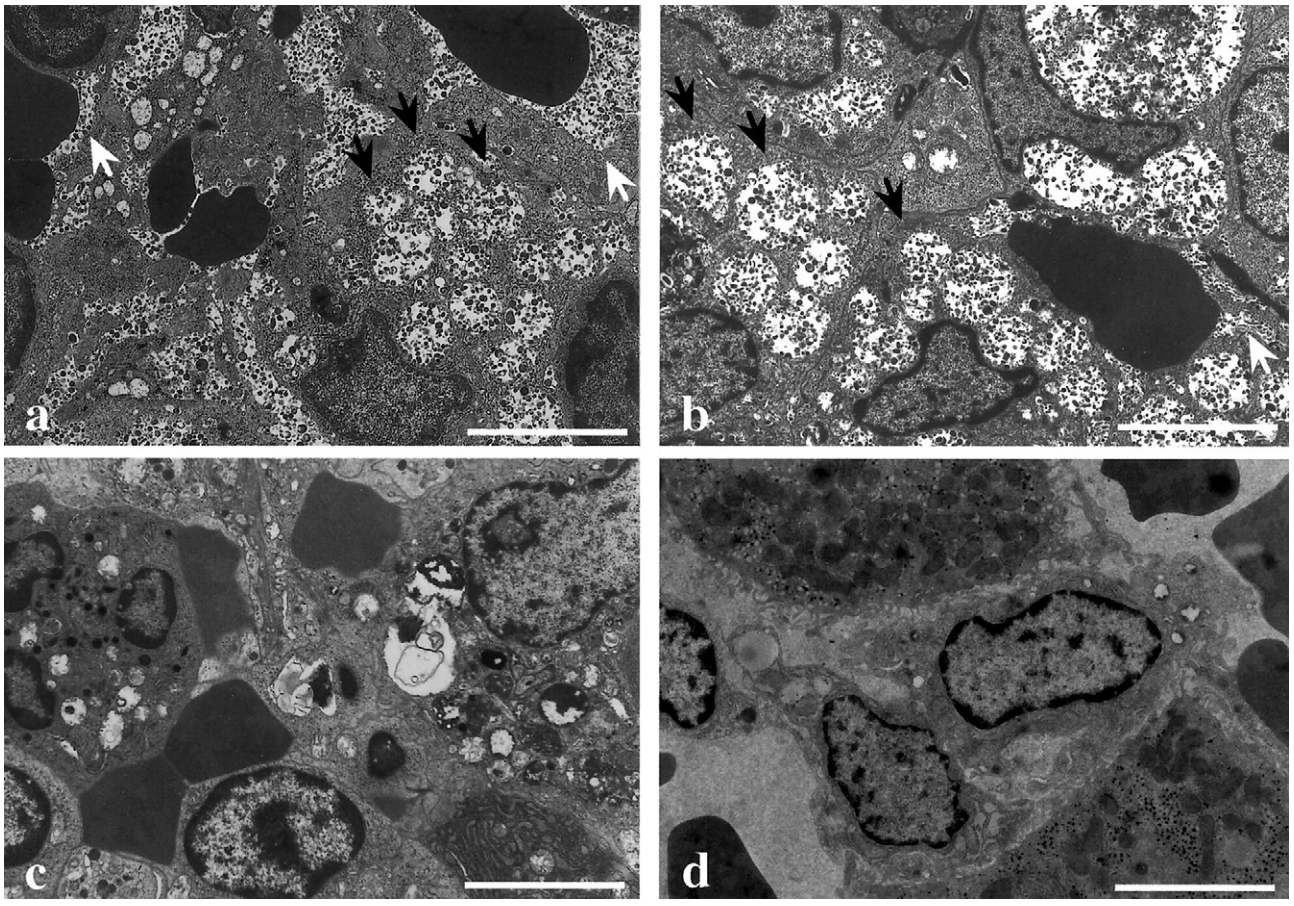

Figure 8. Transmission electron microscopy of spleen (**a, c**) and liver (**b, d**), 1 (**a, b**) and 7 (**c, d**) days after infusion of HbV (20 ml/kg). The HbV particles are seen in the phagosomes (**black arrows**) and capillaries (**white arrows**) both in the spleen and liver after 1 day. After 7 days, no phagosomes with HbV particles can be observed (**c, d**). Bars, 5 μm.

was reported for other oxygen carriers, i.e., Fluosol-DA[28] and liposomes for cancer therapy.[25] The influence of the transient decrease in phagocytic activity after the infusion of HbV should be investigated in detail, especially in the septic shock model when the defense system in a body is significantly depressed.[16,45] However, the phagocytic activity returned to the original level at 2 weeks. The results of the serum clinical chemistry (AST and ALT) indicate that the level of liver function decreased temporarily and recovered to the normal level within 3 days. Judging from these results, the change of liver was considered transient and functional. The initial body weight loss, especially the 20 ml/kg HbV group one day after the HbV administration, may be related to the decreased appetite since the animals did not fast from food and water. However, the body weight of the HbV groups returned to that of the control groups within 3 days and they grew normally.

In our study, simultaneous splenomegaly was observed for the HbV high dose group 3 days after infusion which corresponded to the decreased plasma concentration of HbV (half life of HbV: 20~30 hours). However, the spleen weight did not return to the control value in 2 weeks despite the fact that the phagocytic activity returned to the control value. Histopathological examination indicated that the HbV particles were metabolized within one week, and there was no infarcts in the spleen, indicating that the sustained splenomegaly is not due to the remaining non-metabolized HbV elements but due to the increased amount of phagocytic or parenchymal cells and/or red blood cells.

The administration of lipid microspheres (Intralipid^{TM}) did not greatly influence the phagocytic activity and spleen weight. We selected the lipid microspheres as a control injection according to some reports which showed that lipid microspheres suppress the RES function.[21,24,46,47] The reasons for the small amount of change should be that the total concentration of solutes in a lipid emulsion (10%) is lower than that in the HbV suspension (16 wt %), and that the metabolic route of the HbV is different from that of the lipid microspheres. It was reported that the lipid emulsions were mainly consumed by muscle and fat, with a negligible contribution from the liver and spleen.[48,49]

The histopathological examination clearly demonstrated that the HbV accumulated in the liver was localized in the Kupffer cells. Hepatocytes appeared normal and lobular architecture was not affected by the HbV administration. This accumulation subsided gradually during the experimental period. The HbV accumulated in spleen was localized in macrophages in the red pulp and marginal zone. Uptake of HbV in the spleen may occur

using a filtration mechanism[50] and opsonization.[51] There was no aggregation of HbV particles in renal and pulmonary capillaries at any time. Rudolph et al[52] indicated small pulmonary and glomerular infarcts due to the trapping of large clusters of aggregated particles of the liposome-encapsulated Hb. The results of this study reflect the differences in the physicochemical characteristics of the vesicles. The particle diameter of our HbV is well-regulated to about 250 nm by the extrusion method, and the vesicular surface is modified with PEG to suppress the intervesicular aggregation[34] and this may effectively prevent embolization of capillaries and venules in microcirculation.[35, 36]

Transmission electron microscopy (TEM) one day after infusion revealed that the HbV particles were captured in the macrophages in the spleen and Kupffer cells by endocytosis and not by fusion because the intact HbV particles were present in the phagosomes. The presence of HbV near the RBC indicates the circulating HbV in vasculature. The vesicular structure of HbV completely disappeared at 7 days indicating that they are totally destroyed in the phagosomes. TEM was a very effective tool for detecting the HbV particles in tissues. The conventional method to detect liposomes (phospholipid vesicles) required encapsulation of colloid gold as a microscopic marker.[53, 54] On the other hand, the high electron density of HbV due to the highly concentrated Hb solution in the inner aqueous phase of HbV as well as in RBC provided sufficient contrast of the particle and clearer images than those displayed in other reports to our knowledge.

Anti-human Hb antibody staining was very effective for staining the human Hb-based oxygen carriers in animal tests. The antibody recognizes the globin chain of human Hb in HbV and there is no cross-reaction with rat Hb. The stained portion mostly disappeared within 7 days both in the spleen and liver. 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 [13] and in the spleen.[55] Normally, iron from a heme is stored in the ferritin molecule.[56] This protein has 24 subunits and encloses as many as 4500 iron atoms in the form of an aggregate of ferric hydroxide. 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. It was reported that polymerized Hb were captured by the Kupffer cells and then hemosiderin was confirmed, and the renal tubules showed siderosis.[14] On the other hand, in our HbV study, there were slight Berlin blue stains only in spleen and liver after 3 and 7 days, and they completely disappeared after 14 days. As the anti-human Hb antibody staining disappeared, trace staining of hemosiderin was recognized and it completely disappeared after 14 days. This indicates that the amount of the stored hemosiderin was minimal, thus the metabolism of heme and recycling of iron molecule are suggested to be on the physiological pathway.

As for the membrane components of HbV, it was reported that the infused lipid components of liposomes are entrapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids' and in exhaled air.[57–59] Liposomal cholesterol reappears in blood as lipoprotein cholesterol after entrapment in the Kupffer cells, and then is excreted in bile after entrapment of the corresponding lipoprotein by the parenchymal hepatocytes.[60] Even though the amount of the infused HbV is much greater than in these studies, the main vesicular components of HbV, the phospholipids and cholesterol, would gradually be metabolized in the same manner. There were small amounts of trace staining with oil red O only in liver 3 days after infusion. This indicated that the deposition of neutral lipid components such as glycerides, which may be produced during the metabolism, was minimal. However, further study is necessary to clarify the influence of a large dose of HbV, especially on a lipemic model.

The long-term stability of HbV during storage has been a major issue for the practical use of oxygen carriers especially for emergency situations. In the previous reports the polymerization of phospholipids bearing dienoyl groups in the bilayer membrane of HbV has shown the enormous stabilization of HbV against long term storage in a freezer or as a freeze-dried powder.[61–63] However, the polymerized phospholipid is difficult to decompose or metabolize and the bilayer structure remained even after 30 days with showing cytotoxicity.[64] After infusing polymerized phospholipid, the phagocytic activity remained suppressed even after 3 days, and the recovery was observed after 7 days.[26] On the other hand, in our method, the storage stability of our HbV is achieved by the surface modification with PEG chains and deoxygenation.[6] Accordingly, our PEG-modified HbV shows rapid metabolism once trapped by RES. The PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which is known as an inert macromolecule, should be excreted in the urine through the kidneys.[65] Our HbV possesses a high encapsulation efficiency of Hb, so that the weight ratio of Hb to the total lipid component is nearly 1.6 to 1.8. This is much higher than the previously reported liposome encapsulated Hb. The reduced amount of lipid is beneficial for reducing the burden on RES. Even though several groups have tried so called liposome encapsulated Hb without polymerized phospholipid, there are few reports on the metabolism and the fate of the components. Sakaguchi et al[27] tested the metabolism of their product, Neo Red Cells, histopathologically with the whole body autoradiograms. However, the concentration of Hb and infusion amount were lower than our case. Therefore, our study demonstrates more detailed information with a larger dose rate of HbV for a longer period of observation.

In conclusion, the components of the infused HbV (20 ml/kg i.v.) entrapped in the RES were smoothly metabolized within one week. Even though the HbV infusion modified the phagocytic activity for two weeks, it does not seem to cause any irreversible damage to the phagocytic organs. Our results are demonstrated only in healthy rats, while rats in hemorrhagic shock or septic shock, or with an inflammation reaction, may react differently in these

pathological situations. The infused amount in this study is equal to about 1.4 l in a 70-kg man and larger volume is needed for the further evaluation. Hence safety concerns cannot be totally eliminated by this study. However, our results offer important information for evaluating the safety issues of HbV for clinical use.

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