Hemoglobin Vesicles, Polyethylene Glycol (PEG)ylated Liposomes Developed as a Red Blood Cell Substitute, Do Not Induce the Accelerated Blood Clearance Phenomenon in Mice

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ABSTRACT:
The hemoglobin vesicle (HbV) is an artificial oxygen carrier encapsulating a concentrated hemoglobin solution in a liposome of which the surface is covered with polyethylene glycol (PEG). It was recently reported that repeated injections of PEGylated liposomes induce the accelerated blood clearance (ABC) phenomenon, in which serum anti-PEG IgM plays an essential role. To examine this issue, we investigated whether HbV induces the ABC phenomenon in mice at a dose of 0.1 mg Hb/kg, a dose that is generally known to induce the ABC phenomenon, or at 1400 mg Hb/kg, which is proposed for clinical use. At 7 days after the first injection of nonlabeled HbV (0.1 mg Hb/kg), the mice received HbV in which the Hb had been labeled with $^{125}$I. After a second injection, HbV was rapidly cleared from the circulation, and uptake clearances in liver and spleen were significantly increased. In contrast, at a dose of 1400 mg Hb/kg, the pharmacokinetics of HbV was negligibly affected by repeated injection. It is interesting to note that IgM against HbV was produced 7 days postinjection at both of the above doses, and their recognition site was determined to be 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-PEG in HbV. These results suggest that a clinical dose of HbV does not induce the ABC phenomenon, and that suppression of ABC phenomenon is caused by the saturation of phagocytic processing by the mononuclear phagocyte system. Thus, we conclude that induction of the ABC phenomenon would not be an issue in the dose regimen used in clinical settings.

It is well known that liposomes are able to function as carriers of drugs (Noble et al., 2006) and genes (Tuffin et al., 2005), and they have the ability to enhance blood retention and to specifically target encapsulated materials because the cellular structure of such vesicles is able to protect encapsulated materials against degradation and enhance their biodistribution. Many liposome-type drugs such as AmBisome (Astellas Pharma US, Inc., Deerfield, IL) and Doxil (Ortho Biotech, Horsham, PA), which have been approved for use and are currently in clinical use, take advantage of these characteristics. There is now little doubt that liposomes are useful and are in widespread use. To further enhance the quality and efficiency of liposomes, they are frequently modified with polyethylene glycol (PEG) (Veronese and Pasut, 2005). PEGylated liposomes exhibit a prolonged half-life, a higher stability, are water-soluble, have lower immunogenicity and antigenicity, as well as the potential for specific cell targeting. Because of these attributes, the majority of the recently developed lipidosome formulations are modified with PEG (Sakai et al., 2008; Okamura et al., 2009).

However, Dams et al. (2000) and Ishida et al. (2003a) reported that the intravenous injection of PEGylated liposomes causes a second dose of liposomes to lose their long-circulating characteristics and accumulate extensively in the liver when they are administered twice in the same animal [referred to as the accelerated blood clearance (ABC) phenomenon]. In addition, based on reported liposomal pharmacokinetics data, it is clear that several factors (e.g., size, lipid composition, surface modification, and membrane fluidity) influence the circulating time and the distribution to targeting areas (Ishida et al., 2004; Samad et al., 2007). Ishida et al. (2004) showed that the
physicochemical properties of liposomes, such as lipid composition, diameter, surface modification, and dose, can also have an effect on the ABC phenomenon. Moreover, they also found that anti-PEG IgM, produced by the spleen in response to an injected dose of PEGylated liposomes, is involved in the induction of the ABC phenomenon (Ishida et al., 2006a).

Hemoglobin vesicles (HbV) have been developed as a cellular type of oxygen carrier, in which highly concentrated hemoglobin (Hb) is encapsulated in a phospholipid bilayer membrane with PEG. There are some distinct advantages for HbV to exist in a liposomal structure; the oxygen affinity (P50) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (PLP) (Sakai and Tsuchida, 2007). Furthermore, the diameter of HbV liposomes can be tailored to approximately 250 nm, and further modification by PEG leads to an enhanced lifetime in the blood circulation compared with other types of hemoglobin-based oxygen carriers (1/2 for cell-free Hb and PEGylated Hb in rats of 1.5 and 10.9 h, respectively) (Goins et al., 1995; Lee et al., 2006) because the encapsulation of Hb completely suppresses renal excretion, although HbVs in the circulation are eventually captured by phagocytes in the mononuclear phagocyte system (MPS) (Sakai et al., 2001). In fact, our group reported that HbV has a long circulation time in blood as an oxygen carrier in mouse, rat, rabbit, and a hemorrhagic shock model rat (Sou et al., 2005; Taguchi et al., 2009a,b). Because of these unique characteristics, such liposomes show an oxygen transport comparable with red blood cells (Sakai et al., 2008) and also show improved survival in hemorrhagic shock animal models (Sakai et al., 2004b, 2009).

In clinical use, it is expected that repeated high-dose injections would be required, as a red blood cell substitute, in patients with massive hemorrhage. Therefore, the possibility remains that repeated injections of HbV could induce the ABC phenomenon in a clinical situation. If the ABC phenomenon were induced by repeated injections, then the pharmacological action of HbV could be influenced. Therefore, it becomes necessary to characterize the pharmacokinetic properties of HbV after repeated injections at a dose that is routinely used in clinical practice as a red blood cell substitute.

In this study, we investigated whether the first injection of HbV at a low dose (0.1 mg Hb/kg), in which the lipid dose induced the ABC phenomenon, as reported by Ishida et al. (2003a), or a high dose (1400 mg Hb/kg), a dose that is proposed for use in a clinical situation, would be required, as a red blood cell substitute, in patients with massive hemorrhage. Therefore, the possibility remains that repeated injections of HbV could induce the ABC phenomenon in a clinical situation. If the ABC phenomenon were induced by repeated injections, then the pharmacological action of HbV could be influenced. Therefore, it becomes necessary to characterize the pharmacokinetic properties of HbV after repeated injections at a dose that is routinely used in clinical practice as a red blood cell substitute.

Materials and Methods

Materials. An Hb solution, from outdated donated blood, was provided by the Japanese Red Cross Society (Tokyo, Japan) and purified according to a previously described purification method (Sakai et al., 2002). PLP was purchased from Sigma-Aldrich (St. Louis, MO). Powdered 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-O-hexadecyl-N,succinyl-l-glutamate (DHSG) were purchased from Nippon Fine Chemical (Osaka, Japan), and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-PEG (DSPE-PEG; molecular weight of PEG = 5000) was purchased from NOF Co. (Tokyo, Japan). Recombinant human serum albumin (rHSA) was a gift from Nipro Corp. (Osaka, Japan). Iodine-125 as Na125I was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG (Cayman Chemical, Ann Arbor, MI) and peroxidase-labeled affinity purified antibody to mouse IgM (μ) were purchased from Sigma-Aldrich.

Preparation of HbVs. HbVs were prepared under sterile conditions, as previously reported (Sakai et al., 1997). A typical encapsulated Hb (38 g/dl) solution contained 14.7 mM PLP as an allosteric effector to regulate the P50 to 25 to 28 Torr. The lipid bilayer comprised a mixture of DPPC, cholesterol, and DHSG at a molar ratio of 5:5:1, and PEG-DSPE (0.3 mol%). The HbVs were suspended in a physiological saline solution at [Hb] 10 g/dl, filter-sterilized (Dismic; Toyoo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with N2 for storage. The content of lipopolysaccharide was <0.1 EU/ml.

HbV Labeling with 125I. 125I-labeled HbVs (125I-HbVs) were prepared as previously reported (Taguchi et al., 2009a). In a typical preparation, 125I-HbV was prepared by incubating HbV with Na125I in Iodo-Gen (1,3,4,6-tetrahydro-3,6-dioxyphenylglycoluril) and was separated from 125I by passage through a PD-10 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The 125I-HbVs were then sterile-filtered (pore size, 450 nm) to remove aggregates. More than 97% of the iodine was bound to internal Hb in this HbV preparation. Before use in experiments, two different concentrations of 125I-HbV suspensions were prepared by mixing with nonradioabeled HbV to adjust the target Hb concentration (0.1 or 1400 mg Hb/kg). All the suspensions were mixed with rHSA to adjust the albumin concentration of the vesicle suspension medium to 5 g/dl. Under these conditions, the colloid osmotic pressure of the suspension was maintained constant at approximately 20 mm Hg (Sakai et al., 2004b).

The Pharmacokinetic Experimental Protocol. All of the animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. All the mice were given water containing 5 mM sodium iodide (NaI) for the duration of the experiment to avoid any specific accumulation in the glandula thyroidea. Male ddY mice (28–30 g; Japan SLC, Inc., Shizuoka, Japan) were anesthetized using ether and received a single injection of a nonlabeled HbV suspension (0.1 or 1400 mg Hb/kg, 420 μl/30 g) to the tail vein. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received a 125I-HbV suspension to the tail vein under ether anesthesia (the concentration and injected volume were identical to those for the first injection). Each mouse received a total dose of 2 × 106 cpm/50 g 125I activity. At each time after the injection of 125I-HbV, blood was collected from the inferior vena cava under ether anesthesia, and plasma was separated by centrifugation (3000g, 5 min). One percent bovine serum albumin (BSA) and 40% trichloroacetic acid were added to the plasma to remove degraded protein and free 125I, and pellets were obtained by centrifugation (1000g, 10 min). After collecting blood, the animal was sacrificed for excision of organs (kidney, liver, spleen, heart, and lung), which were rinsed with saline and weighed. 125I radioactivity in the plasma and excised organs was determined using a liquid scintillation counter (ARC-5000; Aloka, Tokyo, Japan).

Quantitative Determination of Anti-HbV IgG and IgM. The ddY mice received injections of saline or HbV (0.1 or 1400 mg Hb/kg, 420 μl/30 g b.w.t.) to the tail vein under ether anesthesia. At each time point (days 3, 7, and 10) after injection, blood was collected from the inferior vena cava. Plasma was collected after centrifugation (3000g, 5 min), and the supernatant was subsequently centrifuged to remove intact HbV (50,000g, 30 min) (Sakai et al., 2003). The supernatant collected as the plasma sample and was stored at −80°C until used.

Enzyme-linked immunosorbent assay (ELISA) was used to detect IgG and IgM against HbV using a previously described method, with minor modifications (Wang et al., 2007). The empty vesicles, which contained 475 ng/ml lipids as HbV (comprising DPPC, cholesterol, DHSG, PEG-DSPE at a molar ratio of 5:5:1:0.3) were added to 96-well plates (ImmuNo 96 MicroWell Plate; Nagle Nune International, Rochester, NY). The plates were incubated for 2 h at 25°C. After incubation, the wells were washed three times with a wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). A blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was then added to each well, and the plate was incubated for 2 h at 25°C. After incubation, the wells were washed three times with wash solution, and 100 μl of plasma sample, diluted 1:100 with sample solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, 1% BSA, pH 8.0), was added to the wells. After incubation for 90 min, the wells were washed three times with wash solution, and 100 μl of 0.2% phenylenediamine (1 mg/ml). After incubation, the reaction was terminated.
by adding 100 μl of 1 N H₂SO₄, and the absorbance was measured at 490 nm using a Microplate reader (model 680; Bio-Rad Laboratories, Tokyo, Japan).

Quantitative Determination of Anti-Lipid IgM. A 10-nmol aliquot of each lipid (DPPC, cholesterol, DHSG, or PEG-DSPE) in 50 μl of 100% ethanol was added to 96-well plates (Immuno 96 MicroWell Plate; Nalge Nunc International). The plates were incubated for 4 h at 37°C to dry completely. After incubation, blocking solution was added to each well, and the plate was incubated for 2 h at 25°C. Following processes were identical to those described under Quantitative Determination of Anti-HbV IgG and IgM.

Data Analysis. Pharmacokinetic analyses, after HbV injections, involved the use of a two-compartment model, and pharmacokinetic parameters were estimated by curve fitting. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). The uptake clearance (CLuptake) was calculated as described in a previous report using integration plot analysis at designated times (from 1–30 min), during which time the efflux and/or elimination of radioactivity from tissues were negligible (Murata et al., 1998). Data are shown as mean ± S.D. for the indicated number of animals. The Bonferroni test was used for comparisons with a saline injection group within each group. Significant differences among each group were examined using the Student’s t test. A probability value of \( p < 0.05 \) was considered to indicate statistical significance.

Results

Pharmacokinetic Properties of HbV after Repeated Injection of 0.1 or 1400 mg Hb/kg. The fate of the 125I-HbV administered to mice was evaluated by determining the residual trichloroacetic acid-precipitable radioactivity in plasma. In this study, the time interval for injection was selected for 7 days based on the previous report, in which ABC phenomenon in mice was observed the most strongly when the time interval for the injection was 7 to 10 days (Ishida et al., 2003b). In addition, blood viscosity after high-dose administration of HbV was equal to that before administration of HbV (Sakai et al., 1998), and repeated infusion of HbV had no adverse clinical signs or symptoms (Sakai et al., 2004a). Figure 1 shows the time course for the plasma concentration curve for 125I-HbV administered once or twice to mice, and Table 1 lists the pharmacokinetic parameters obtained using the two-compartment model.

At a dose of 0.1 mg Hb/kg, plasma HbV in the second injection was rapidly cleared compared with that in the first injection (Fig. 1A). The half-life (\( t_{1/2} \)) in the second injection was reduced significantly—by approximately half—compared with that in the first injection. Accompanied by the reduction in \( t_{1/2} \), the area under the concentration-time curve (AUC) was also significantly decreased (27.1 ± 18 and 4.5 ± 3.8 hr% of dose/ml, \( p < 0.001 \), for first and second injection, respectively), whereas plasma clearance (CL) was significantly increased in the second injection compared with that in the first injection (3.69 ± 0.4 and 22.3 ± 8.1 ml/h, \( p < 0.001 \), for the first and second injections, respectively). However, the distribution volume of the central compartment (\( V_d \)) remained unchanged as the result of repeated injections (Table 1).

At a dose of 1400 mg Hb/kg, the values of \( t_{1/2} \) and CL in the second injection were not significantly different from those for the first injection, but the AUC was decreased slightly, in the case of the second injection (829 ± 38 and 695 ± 38 hr% of dose/ml, \( p < 0.05 \), for first and second injections, respectively) (Table 1).

Effect of Repeated Injection on the Hepatic and Splenic Distribution of HbV. Because liver is the major distribution organ for HbV (Taguchi et al., 2009b), the effect of repeated injections on the hepatic distribution of HbV was examined. Figure 2 shows the time course distribution for 125I-HbV (percentage of injection of dose) in the liver after the administration of 125I-HbV once or twice. Up to 0.5 h after the injection of 125I-HbV at a dose of 0.1 mg Hb/kg, the hepatic distribution of 125I-HbV in the second injection was much higher than that in the first injection (Fig. 2A, inset). However, after 0.5 h or more, the differences between the first and second injections were minor (Fig. 2A). From the beginning after an HbV injection at a dose of 1400 mg Hb/kg, hepatic distributions of 125I-HbV were similar between the first and the second injection (Fig. 2B, inset), and this tendency was maintained for periods of up to 72 h.

We next calculated the CLuptake in the liver (Table 2). At a dose of 0.1 mg Hb/kg, the CLuptake for the second injection was 8.5 times higher than that for the first injection (3.5 ± 0.4 and 29.6 ± 18 ml/h, \( p < 0.01 \), for the first and the second injection, respectively), whereas at a dosage of 1400 mg Hb/kg, the CLuptake for the second injection was only 1.5 times higher than that for the first injection (0.26 ± 0.04 and 0.37 ± 0.03 ml/h, \( p < 0.05 \), for the first and the second injection, respectively).

Because the spleen is an another major distribution organ of HbV (Taguchi et al., 2009b) and an essential organ in terms of inducing the ABC phenomenon (Ishida et al., 2006a), we also examined the time course for the distribution of 125I-HbV (percentage of injection of dose) in the spleen. For periods up to 1 h after HbV injection, the splenic distributions of 125I-HbV in the first and the second injections were not greatly different for doses of both 0.1 and 1400 mg Hb/kg (Fig. 3, A and B, insert). However, 1 h or more after the second injection, higher splenic distributions of HbV were observed in both the low- and high-dose groups compared with those in the first injection (Fig. 3, A and B). In addition, we calculated the CLuptake in...
Mice received a single or double injection of $^{125}\text{I}-\text{HbV}$ (0.1 and 1400 mg Hb/kg) containing 5% rHSA. At each time after the $^{125}\text{I}-\text{HbV}$ injection, blood was collected from the inferior vena cava, and plasma was obtained. Each parameter was calculated by MULTI using the two-compartment model. The values are mean ± S.D. ($n$ = 3–6).

### TABLE 1
Pharmacokinetic parameters for HbV after one or two injections of $^{125}\text{I}-\text{HbV}$ in mice

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<thead>
<tr>
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<th>First Injection</th>
<th>Second Injection</th>
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<tr>
<td>$V_1$ (ml)</td>
<td>2.7 ± 0.2</td>
<td>3.1 ± 0.3</td>
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<tr>
<td>AUC (h*% of dose/ml)</td>
<td>27.1 ± 18</td>
<td>4.5 ± 3.8**</td>
</tr>
<tr>
<td>CL (ml/h)</td>
<td>3.69 ± 0.4</td>
<td>22.3 ± 8.1**</td>
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<tr>
<td>$t_{1/2}$ (h)</td>
<td>18.8 ± 1.3</td>
<td>695 ± 38*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.1 ± 0.3</td>
<td>3.2 ± 0.3</td>
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$V_1$, the distribution volume of the central compartment.

* $p < 0.05$, ** $p < 0.001$ vs. first injection.

FIG. 2. Time courses for radioactivity in liver after the first injection (open circles) or the second injection (filled circles) of $^{125}\text{I}-\text{HbV}$ to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or $^{125}\text{I}-\text{HbV}$ to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. At a dose of 0.1 mg Hb/kg, the CLuptake for the second injection was 4.5 times higher than that for the first injection (1.6 ± 0.1 and 7.2 ± 3.2 ml/h, $p < 0.01$, for the first and the second injection, respectively). At a dose of 1400 mg Hb/kg, the CLuptake for the second injection (0.07 ± 0.02 ml/h) was not significantly changed compared with that for the first injection (0.05 ± 0.01 ml/h).

Furthermore, we also examined the distribution of $^{125}\text{I}-\text{HbV}$ in the kidney, lung, and heart at doses of both 0.1 and 1400 mg Hb/kg. No significant differences were observed between the first and the second injections (data not shown).

**Determination of IgG and IgM against HbV after HbV Injection.** In a previous study, it was reported that IgM, which is produced by the preinjection of PEGylated liposomes, is strongly involved in the induction of the ABC phenomenon (Ishida et al., 2006b). Therefore, we examined the issue of whether IgG or IgM against HbV is elicited by an initial injection of saline or HbV at a dose of 0.1 and 1400 mg Hb/kg. Figure 4 shows the quantitative determination of plasma IgG (A) and IgM (B) against HbV. Negligible levels of IgG were elicited against HbV in all the injection groups at 3, 7, and 10 days after the injection of saline or HbV (Fig. 4A). In contrast, the IgM against HbV was significantly elicited starting from 3 days after the first injection of HbV at a dose of 0.1 mg Hb/kg (Fig. 4B). On the other hand, at a dose of 1400 mg Hb/kg, the IgM against HbV was significantly elicited starting from 7 days after the first injection. At 10 days after the first injection, IgM levels against HbV at a dose of 1400 mg Hb/kg were significantly higher than the levels at a dose of 0.1 mg Hb/kg ($p < 0.01$) (Fig. 4B).

**Determination of the Specific Recognition Site of IgM against HbV.** To evaluate the specific recognition site of IgM against HbV, a modified ELISA was employed using each lipid component of HbV. Figure 5 shows data for the quantitative determination of the specific recognition site of IgM against HbV at 3, 7, and 10 days after the first injection of HbV at doses of 0.1 or 1400 mg Hb/kg. At a dose of 0.1 mg Hb/kg, strong binding of IgM to DSPE-PEG was observed, starting at day 3 after the first injection, whereas a dramatic enhancement in the binding of IgM to DSPE-PEG was observed, starting at 7 days at a dose of 1400 mg Hb/kg. On the other hand, IgM against other lipid components (DPPC, cholesterol, and DHSG) were negligible during all the times examined after the injection of both low and high doses of HbV.

**Discussion**

As discussed in the introduction, HbV is a red blood cell substitute, the proposed dose of which is 1400 mg Hb/kg. This dosage is more than 100 times higher than that of liposome preparations used as pharmaceuticals, and the use of multiple doses is planned under clinical situations. Therefore, an investigation of whether repeated HbV injections induce the ABC phenomenon is a necessity. However, little information is available on the ABC phenomenon at such extraordinarily high doses of liposomes. In this study, we found an interesting phenomenon, namely, that repeated injections of HbV to mice at a dose of 1400 mg Hb/kg did not seem to induce the ABC phenomenon, even though the plasma levels of IgM against HbV were significantly elevated.

When mice received injections of a low-dose (0.1 mg Hb/kg) HbV, a dose that Ishida et al. (2003b) reported induced the ABC phenom-
The ABC phenomenon was clearly induced at 7 days postinjection (Fig. 1A; Table 1). Consequently, the pharmacokinetics of HbV was markedly changed. For example, the $t_{1/2}$ and AUC for HbV in the second injection were significantly decreased compared with the values for the first injection, and the CL for the second injection was significantly increased. In addition, the hepatic distribution of $^{125}$I-HbV after the second injection at a dose of 0.1 mg Hb/kg was increased for periods of up to 30 min (Fig. 2A) with an increase in hepatic uptake clearance for the second injection (Table 2).

A previous study, Dams et al. (2000) reported that, in mice that were administered liposomes at weekly intervals at a dose of 5 μmol of phospholipids/kg, the ABC phenomenon was not induced. It is well known that a variety of factors, including the lipid dose and physicochemical properties (degree of PEGylation, PEG chain length, surface charge and size) of the initially injected liposome, strongly affect the pharmacokinetic response to subsequent injection (Ishida et al., 2004). For example, it appears that the ABC phenomenon was not caused by preinjection with smaller-sized polymeric micelles but was triggered by preinjection with larger-sized polymeric micelles (Koide et al., 2008). Wang et al. (2005) found that the induction and magnitude of the ABC phenomenon were also influenced by the lipid composition.

### TABLE 2

Uptake clearance of HbV in the liver and spleen of mice receiving injections of $^{125}$I-HbV

All of the mice received a single or double injection of $^{125}$I-HbV (0.1 and 1400 mg Hb/kg) containing 5% rHSA. The uptake clearance for each organ was calculated by integration plot analysis at designated times from 1 to 30 min after injection. The values are mean ± S.D. ($n = 3–6$).

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<th>0.1 mg Hb/kg</th>
<th>1400 mg Hb/kg</th>
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<tr>
<td></td>
<td>First Injection</td>
<td>Second Injection</td>
</tr>
<tr>
<td>Liver (ml/h)</td>
<td>3.5 ± 0.4</td>
<td>29.6 ± 18*</td>
</tr>
<tr>
<td>Spleen (ml/h)</td>
<td>1.6 ± 0.1</td>
<td>7.2 ± 3.2*</td>
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* $p < 0.01$ and ** $p < 0.05$ vs. first injection.

**Fig. 3.** Time courses for radioactivity in spleen after the first injection (open circles) or the second injection (filled circles) of $^{125}$I-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or $^{125}$I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the $^{125}$I-HbV suspension to the tail vein. Each point represents the mean ± S.D. ($n = 3–6$).

**Fig. 4.** Determination of IgG (A) and IgM (B) against HbV after a single intravenous injection of saline (open bars), HbV at a dose of 0.1 mg Hb/kg (gray bars) or 1400 mg Hb/kg (closed bars) in mice. The ddY mice received injections of saline or HbV (0.1 or 1400 mg Hb/kg) to the tail vein. At 3, 7, and 10 days after injection of saline or HbV, blood was collected from the inferior vena cava, and plasma was obtained. Anti-HbV IgG and IgM were detected with ELISA. Each bar represents the mean ± S.D. ($n = 4$). **, $p < 0.01$. ** 
In contrast to the low-dose treatment, the injection of a high dose (1400 mg Hb/kg) of HbV did not appear to induce the ABC phenomenon in mice (Fig. 1B; Table 1). In fact, the hepatic uptake clearance for the second injection was only 1.5 times higher than that for the first injection (Table 2). Ishida et al. (2004) previously reported that the lipid dose of a prior injection of liposomes strongly affected the pharmacokinetic behavior of a subsequent injection at a dose of 0.001 to 25 μmol of phospholipids/kg. They reported that liver accumulation in mice increased sigmoidally with increasing lipid dose, whereas the blood concentration sigmoidally decreased with increasing lipid dose. In general, the increased hepatic or splenic distributions of liposomes were accompanied by an increased scavenging of liposome by MPS, such as Kupffer cells and red pulp zone splenocytes (Goins et al., 1995). MPS or any other systems that are involved in the removal of liposomes are influenced by the injection dose (Laverman et al., 2000), and the uptake by MPS was saturated with increasing doses of liposomes. Our previous study showed that the distribution of HbV in the liver was saturated at 1400 mg Hb/kg (more than 100 μmol of phospholipids/kg) but not at 200 mg Hb/kg (approximately 25 μmol of phospholipids/kg) (Taguchi et al., 2009b).

Consequently, HbV at a dose of 1400 mg Hb/kg did not appear to induce the ABC phenomenon, even though accompanied with remarkable IgM elicitation, because the hepatic uptake of HbV via MPS was saturated in the case of a high-dose injection. From these results, it was expected that ABC phenomenon might not be apparently induced at various intervals at proposed dose of HbV (1400 mg Hb/kg) because ABC phenomenon in mice was observed the most strongly at the 7- to 10-day interval (Ishida et al., 2003b). In fact, we previously reported that ABC phenomenon was not induced in hemorrhagic shock model rat, when HbV was injected at a dose of 1400 mg Hb/kg at hourly intervals (Taguchi et al., 2009a), at which the patients with massive hemorrhage are transfused.

However, our study has limitations with respect to explanation of full-length study of the ABC phenomenon of HbV. We have not examined the plasma IgM levels when multiple high doses of HbVs were administered. Dams et al. (2000) previously reported that weekly injections of N-hydroxysuccinimidyl hydrazino nicotinate hydrochloride PEG liposomes dramatically influenced the circulatory half-life at second injection, but the effect was almost normalized at fourth injection. Therefore, it seems that the higher levels of IgM elevations are not observed after multiple high-dose administration of HbV. On this point, further study could be needed for elucidating the effect of multiple high-dose administration of HbV on their pharmacokinetics.

In conclusion, the present study clearly shows that repeated injections of HbV induce the ABC phenomenon, when the first injection of HbV was a dose of 0.1 mg Hb/kg, but was not apparent at a dose of 1400 mg Hb/kg. These results suggest that, in a clinical situation, the repeated use of HbV at a dose of 1400 mg Hb/kg would not be expected to induce the ABC phenomenon. Thus, it is unlikely to be necessary to consider the ABC phenomenon in an administration schedule or regimen of HbV treatment as a red blood cell substitute.

References


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