Repeated Injection of High Doses of Hemoglobin-Encapsulated Liposomes (Hemoglobin Vesicles) Induces Accelerated Blood Clearance in a Hemorrhagic Shock Rat Model

Kazuaki Taguchi, Yasunori Iwao, Hiroshi Watanabe, Daisuke Kadowaki, Hiromi Sakai, Koichi Kobayashi, Hirohsa Horinouchi, Toru Maruyama, and Masaki Otagiri

Department of Biopharmaceutics (K.T., Y.I., H.W., D.K., T.M., M.O.), Center for Clinical Pharmaceutical Sciences (H.W., D.K., T.M.), Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan; Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan (M.O.); Research Institute for Science and Engineering, Waseda University, Tokyo, Japan (H.S.); and Department of Surgery, School of Medicine, Keio University, Tokyo, Japan (K.K., H.H.)

Received October 24, 2010; accepted December 1, 2010

ABSTRACT:

The hemoglobin vesicle (HbV) is an artificial oxygen carrier in which a concentrated hemoglobin solution is encapsulated in a liposome. To apply liposome preparations in clinics, it is important to consider the accelerated blood clearance phenomenon (ABC phenomenon), which involves a loss in the long-circulation half-life after being administered repeatedly to the same animals. The objective of this study was to determine whether the ABC phenomenon is induced by repeated injection of HbV under conditions of hemorrhagic shock. We created a rat model of hemorrhagic shock and performed a pharmacokinetic study using $^{125}$I-HbV, in which the Hb inside of HbV was labeled with $^{125}$I. At 4 and 7 days after resuscitation from hemorrhagic shock by nonlabeled HbV (1400 mg Hb/kg), the second dose of $^{125}$I-HbV (1400 mg Hb/kg) was rapidly cleared from the circulation compared with normal rats. Of interest, IgM against HbV was produced at 4 days after the first injection of HbV, but decreased at 7 days. In addition, phagocyte activity was increased at both 4 and 7 days after the first injection of HbV. These results suggest that repeated injections of HbV at a dose of 1400 mg Hb/kg induce the ABC phenomenon under conditions of hemorrhagic shock, which is strongly related to both the production of anti-HbV IgM and enhanced phagocyte activity. We thus conclude that it might be necessary to consider the ABC phenomenon in the dose regimen of HbV treatment in clinical settings.

Introduction

Hemoglobin-based artificial oxygen carriers (HBOCs), which include cross-linked (Chen et al., 2009), polymerized (Jahr et al., 2008), and polymer-conjugated Hb (Smani, 2008), have been developed to overcome problems associated with blood transfusion, such as cross-matching, blood-bone infections (human immunodeficiency virus and hepatitis virus), and the shortage of donated blood. Several of these HBOCs are currently in the final stages of clinical evaluation. However, Natsanson et al. (2008) recently performed a meta-analysis based on data from randomized controlled trials of five different acellular-type HBOCs and concluded that acellular-type HBOCs are associated with a significantly increased risk of death and myocardial infarction. This risk would be induced by the scavenging of nitric oxide (NO) by cell-free Hb, because it was reported that a reduction in NO levels in myocardial lesions is an important factor in inducing histological damage in cases of myocardial lesions (Burhop et al., 2004).

The hemoglobin vesicle (HbV) is an artificial oxygen carrier with a cellular structure (liposome structure) similar to that of red blood cells (RBCs): highly concentrated Hb encapsulated in a phospholipid bilayer membrane with polyethylene glycol (PEG). Because this membrane reduces interactions between Hb and NO, adverse effects, such as hypertension and histological damage in myocardial lesions, are not induced, as are found for acellular-type HBOCs (Sakai et al., 2000, 2004a). In addition, there are some distinct advantages associated with the membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an enhanced membrane structure of HbV as follows; the oxygen affinity (P$_{50}$) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5'-phosphate (Sakai and Tsuchida, 2007), an expanded lifetime in the blood circulation compared with other types of HBOCs (Sou et al., 2005; Taguchi et al., 2009c), guarantees long-term storage for periods of more than 2 years at room temperature (Tsuchida et al., 2009). Moreover, HbV possesses oxygen transport characteristics that are comparable to those of RBCs. In fact, the pharmacological effects of HbV have been reported to be equivalent to that of RBCs, when injected into hemorrhagic shock animals (Sakai et al., 2004b, 2009). Therefore, HbV has attracted considerable attention as a potential candidate for use as an artificial oxygen carrier and has considerable promise for use in clinical settings.

ABBREVIATIONS: HBOC, hemoglobin-based artificial oxygen carrier; NO, nitric oxide; HbV, hemoglobin vesicle; RBC, red blood cell; PEG, polyethylene glycol; ABC phenomenon, accelerated blood clearance phenomenon; SD, Sprague-Dawley; HS, hemorrhagic shock; MZ, marginal zone.
It was reported that PEGylated liposomes showed some unexpected pharmacokinetic properties, the so-called accelerated blood clearance phenomenon (ABC phenomenon), in which the long-circulation half-life is lost after liposomes are administered twice to the same animals (Laverman et al., 2001; Ishida and Kiwada, 2008). Ishida et al. (2006a) proposed a mechanism for the ABC phenomenon as follows. IgM, produced in the spleen by the first injection of PEGylated liposomes, selectively binds to the second injected PEGylated liposomes and subsequent complement activation by IgM results in accelerated clearance and enhanced hepatic uptake of the second injected dose of PEGylated liposomes. In the case of HbV, there have been several explanations for the induction of the ABC phenomenon as follows: 1) HbV has a liposome structure that contains PEG; 2) our previous study, using normal mice, showed that the ABC phenomenon was not induced, but anti-HbV IgM was produced 7 days after the injection of HbV at a dose of 1400 mg Hb/kg (Taguchi et al., 2009c); and 3) the pharmacokinetic properties of HbV are altered under the various pathological conditions (Taguchi et al., 2009a, 2010). Therefore, it is possible that the pharmacokinetics of HbV become altered by repeated administration in various pathological conditions. In a clinical setting, HbV would be used to treat a massive hemorrhage, and repeated administrations would be required. If the pharmacokinetics of HbV were altered as the result of repeated injections, then the pharmacological action of HbV would probably be influenced. Therefore, it becomes necessary to clarify the pharmacokinetics associated with the repeated injection of HbV under conditions of massive hemorrhage.

The objective of the present study was to investigate whether the ABC phenomenon is induced by repeated injection of HbV under conditions of massive hemorrhage. To accomplish this, we examined changes in the pharmacokinetics of HbV, using 125I-HbV [the internal standard of 125I] during repeated administration using a rat model of hemorrhagic shock. In addition, we further studied the mechanism of the induction of the ABC phenomenon under our experimental conditions.

Materials and Methods

Preparation of HbV. HbV was prepared under sterile conditions as reported previously (Sakai et al., 1997). In brief, an Hb solution was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mg pyridoxal 5'-phosphate (Sigma-Aldrich, St. Louis, MO) as an allosteric effector to maintain the P50 to 25–28 Torr. The lipid bilayer was a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-O-hexadecyl-N-succinyl-l-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan) at a molar ratio of 5:5:1, and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-N-PEG (NOF Corp., Tokyo, Japan) (0.3 mol %). The size of the HbV particles was controlled at approximately 250 nm by the extrusion method used. The HbV was suspended in a physiological salt solution at [Hb] 10 g/dl, filter-sterilized (pore size 450 nm; Dismic, Toyo-Roshi, Tokyo, Japan) and bubbled with N2 for storage. The lipopolysaccharide content was <0.1 endotoxin unit/ml.

Before all experiments, HbV was mixed with recombiant human serum albumin (Nipro Corp., Osaka, Japan) to adjust the albumin concentration of the suspension medium to 5 g/dl. Under these conditions, the colloid osmotic pressure of the suspension can be kept constant at approximately 20 mm Hg (Sakai et al., 2004b).

Preparation of Hemorrhagic Shock Model Rats. All animal experiments were performed according to the guidelines, principles, and procedures of Kumamoto University for the care and use of laboratory animals. SD rats were maintained in a temperature-controlled room with a 12-h dark/light cycle and ad libitum access to food and water. Hemorrhagic shock model rats were prepared as described in a previous report (Taguchi et al., 2009a). Hemorrhagic shock was induced by removal of 40% of the total blood volume (22.4 ml/kg).

The systemic blood volume was estimated to be 56 ml/kg (Sakai et al., 2004b). After removal of the blood, the hemorrhagic shock rats were resuscitated by an infusion of isovolemic HbV (1400 mg Hb/kg, 22.4 ml/kg). After resuscitation, all rats were housed in a temperature-controlled room with a 12-h dark/light cycle with ad libitum access to food and water.

Quantitative Determination of Anti-HbV IgG and IgM. Five SD rats with hemorrhagic shock were resuscitated with isovolemic HbV (1400 mg Hb/kg, 22.4 ml/kg). Every day after injection, blood was collected from the tail vein under ether anesthesia. Plasma was collected after centrifugation (3000g, 5 min) and subsequently ultracentrifuged (PEGV, 100000 g, 30 min) (Sakai et al., 2003). The supernatant was collected as the plasma sample and was stored at −80°C until used. The IgG and IgM against HbV were detected as described in a previous report (Taguchi et al., 2009c).

Pharmacokinetic Experiments. 125I-HbV was prepared as described in a previous report (Taguchi et al., 2009b). In short, 125I-HbV was prepared by incubation of HbV with Na125I (PerkinElmer Life and Analytical Sciences, Waltham, MA) in an Iodogen (1,3,4,6-tetrachloro-3a,4a-diphenylglycoluril) tube for 30 min at room temperature. 125I-HbV was then isolated from free 125I by passage through a PD-10 column (GE Healthcare, Uppsala, Sweden). More than 97% of the total iodine was bound to the internal Hb in HbV. All suspensions were mixed with recombiant human serum albumin (5 g/dl).

All rats were given water containing 5 mM sodium iodide for the duration of the experiment to avoid specific accumulation in the glandula thyreoidea. Ten SD rats were induced with hemorrhagic shock and resuscitated with HbV, and the pharmacokinetic study was performed at 4 days (n = 5) or 7 days (n = 5) after resuscitation. Normal rats (n = 5) were also used as controls. All rats were anesthetized with pentobarbital, and polyethylene catheters were inserted into the left femoral vein. After infusion of 125I-HbV (1400 mg Hb/kg), blood samples were collected at multiple time points after the 125I-HbV injection (3 min, 10 min, 30 min, 1 h, 6 h, 12 h, and 24 h) and the plasma was separated by centrifugation (3000g, 5 min). Degraded HbV and free 125I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% trichloroacetic acid. After collection of the final blood samples (24 h), the rats were euthanized, and the organs were excised (kidney, liver, spleen, lung, and heart), rinsed with saline, and weighed. The levels of 125I in the plasma and excised organs were determined using a gamma counter (ARC-5000; Aloka, Tokyo, Japan).

Determination of Total Blood Volume. Total blood volume was determined using the Evans blue dilution technique as described previously, with minor modifications (Kuebler et al., 2004). In brief, 4 or 7 days after resuscitation, the rats received an intravenous bolus of 1 mg of Evans blue dye in 1 ml of normal saline. At 2 min after injection, blood samples (1 ml) were collected. The samples were centrifuged, and the absorbance of each sample was measured at 620 and 750 nm. The concentration of Evans blue was determined using a standard curve of Evans blue in excess plasma in correlation to the extinction at 620 nm corrected for turbidity at 750 nm. Total blood volume was calculated using the following formula: total blood volume = total plasma volume/100% − hematocrit (percent) × (0.01) (Clavijo-Alvarez et al., 2005).

Measurements of Phagocyte Activity. Phagocyte activity was determined by the carbon clearance method, as described in a previous report (Sakai et al., 2001; Taguchi et al., 2010). Ten SD rats were induced with hemorrhagic shock and resuscitated with HbV, and carbon clearance was determined 4 days (n = 5) or 7 days (n = 5) after resuscitation. Normal healthy rats without HbV injection (n = 5) were also used as controls. In a typical experiment, rats were anesthetized with pentobarbitral. Polyelectrolyne catheters (PE-50 tubing) containing saline and heparin were then introduced into the left femoral vein for the infusion of a carbon particle solution and for blood collection. The carbon particle solution (Fount India Ink; Pelikan, Hannover, Germany) was infused at 10 ml/kg within 1 min. At 4, 10, 20, 30, 45, and 60 min later, approximately 100 µl of blood was then withdrawn, and precisely a 50-µl aliquot was diluted with 5 ml of a 0.1% sodium bicarbonate solution. The absorption was measured at 675 nm by means of a spectrophotometer (U-2900; Hitachi, Tokyo, Japan). The phagocyte index (K) was calculated using the equation $K = l(t_2 - t_1) / \ln(C_1/C_2)$, where $C_1$ and $C_2$ are the concentrations (absorbance) at time $t_1$ and $t_2$ (min), respectively.

Measurement of Carbon Activity (CH50). Ten SD rats were induced with hemorrhagic shock and resuscitated with HbV, and blood samples were col-
lected at 4 or 7 days after resuscitation. The blood was centrifuged (3000g, 5 min) to obtain plasma for analysis. All plasma samples were stored at \(-80^\circ\text{C}\) before analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan). The CH50 was detected by the method of Mayer (1961).

**Data Analysis.** Data are shown as the mean \(\pm\) S.D. for the indicated number of animals. Significant differences among each group were determined using the two-tail unpaired Student's \(t\) test. Pharmacokinetic analyses after HbV administration proceeded on the basis of a two-compartment model. Pharmacokinetic parameters were calculated by fitting using MULTI, a normal least-squares program (Yamaoka et al., 1981). A probability value of \(p < 0.05\) was considered to indicate statistical significance.

**Results**

**Production of Anti-HbV IgG and IgM.** In a previous study, it was reported that anti-liposome IgM, 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 anti-HbV IgG and IgM are produced by an initial injection of HbV at a dose appropriate for clinical use (1400 mg Hb/kg) in the rat model of hemorrhagic shock. As shown in Fig. 1, the levels of anti-HbV IgG were negligibly increased after the injection of HbV. In contrast, anti-HbV IgM was elicited starting at 3 days after resuscitation by HbV. The highest value was found at 4 days and gradually decreased until 7 days after the injection of HbV. These results suggest that repeated injection of HbV might induce the ABC phenomenon, even under conditions of hemorrhagic shock. The following experiments were performed at the time points of 4 days (HS4 day) and 7 days (HS7 day) after resuscitation by HbV.

**Pharmacokinetic Study.** The fate of the 125I-HbV administered to normal, HS4 day, and HS7 day rats was evaluated by determining residual trichloroacetic acid-precipitable radioactivity in the plasma. Figure 2A shows the time course for the plasma concentration of 125I-HbV in normal, HS4 day and HS7 day rats, and Table 1 lists the pharmacokinetic parameters for these groups. Plasma retention in the HS4 day and HS7 day rats decreased rapidly compared with that in normal rats, and the plasma clearance of 125I-HbV in the HS4 day and HS7 day rats was 1.7- and 1.9-fold increased compared with that in normal rats (Table 1). Accompanied by a decrease in clearance, the area under the time-concentration curve was also significantly decreased by half, whereas the elimination-phase half-life of 125I-HbV was also significantly deceased in the hemorrhagic shock model rats compared with normal rats. The pharmacokinetic parameters were not significantly different between the HS4 day and HS7 day rats.

Figure 2B shows the tissue distribution of 125I-HbV (percentage of injected dose) at 24 h after 125I-HbV administration. Similar to normal rats, in the HS4 day and HS7 day rats 125I-HbV was mainly distributed in the liver and spleen. However, the amount of 125I-HbV distribution in the liver was significantly increased in the HS4 day and HS7 day rats compared with that in normal rats, whereas that in the spleen was not significantly different among the three groups. These data indicate that the ABC phenomenon is induced in HS4 day and HS7 day rats.

**FIG. 1.** The production of anti-HbV IgG and IgM after resuscitation by HbV in a rat model of hemorrhagic shock. Hemorrhagic shock was induced in SD rats with normal, HS4 day and HS7 day rats. The blood was collected from the tail vein, and plasma was obtained. Anti-HbV IgG and IgM were detected with an enzyme-linked immunosorbent assay. Each bar represents the mean \(\pm\) S.D. (n = 5).

**Fig. 2.** A, plasma concentration curve of 125I-HbV after administration to normal (○), HS4 day (□), and HS7 day (●) rats at a dose of 1400 mg Hb/kg. B, tissue distributions of 125I-HbV at 24 h after administration to normal (○), HS4 day (□), and HS7 day (●) rats at a dose of 1400 mg Hb/kg. All rats received 125I-HbV at a dose of 1400 mg Hb/kg; blood samples were collected at multiple time points (3 min, 10 min, 30 min, 1 h, 6 h, 12 h, and 24 h), and plasma samples were obtained. After collection of the final blood sample, each organ was collected at 24 h after injection. Each point represents the mean \(\pm\) S.D. (n = 5). ***, \(p < 0.01\) versus normal rats.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>4 day</th>
<th>7 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2(t12h) (h)</td>
<td>5.3 (\pm) 3.9</td>
<td>0.53 (\pm) 0.07*</td>
<td>0.47 (\pm) 0.22*</td>
</tr>
<tr>
<td>1/2(t12h) (h)</td>
<td>30.4 (\pm) 6.0</td>
<td>22.3 (\pm) 3.5*</td>
<td>22.0 (\pm) 3.2*</td>
</tr>
<tr>
<td>Ke ((\times)105 min(^{-1}))</td>
<td>0.70 (\pm) 0.06</td>
<td>1.40 (\pm) 0.38*</td>
<td>1.25 (\pm) 0.18*</td>
</tr>
<tr>
<td>AUC (b % of dose/ml)</td>
<td>210.3 (\pm) 22.9</td>
<td>115.9 (\pm) 24.1*</td>
<td>129.4 (\pm) 12.1*</td>
</tr>
<tr>
<td>CL (ml/h)</td>
<td>0.47 (\pm) 0.04</td>
<td>0.90 (\pm) 0.21*</td>
<td>0.78 (\pm) 0.07*</td>
</tr>
</tbody>
</table>

1/2(t12h), the distribution-phase half-life; 1/2(t12h), the elimination-phase half-life; AUC, area under the concentration-time curve; CL, clearance.

* \(p < 0.01\) versus normal.
HS7 day rats, and this would be accompanied by an increased distribution in the liver.

**Measurement of Total Blood Volume.** It was previously observed that the retention of HbV in the circulation was decreased when the systemic blood volume decreased (Taguchi et al., 2009a). Therefore, we measured the total blood volume in normal, HS4 day, and HS7 day rats using the Evans blue dilution technique. As shown in Fig. 3, the total blood volume in the HS4 day rats was significantly changed, but this change was not remarkable compared with the massive bleeding. These data indicate that the shorter retention in the circulation in the HS4 day and HS7 day rats was not due to a decreased systemic blood volume and that other factors are strongly involved in this phenomenon.

**Complement Activity.** It is well known that the ABC phenomenon is induced by the selective binding of anti-liposome IgM to the second injected PEGylated liposomes, and subsequent complement activation by IgM results in accelerated clearance and enhanced hepatic uptake of the second injected PEGylated liposomes (Ishida and Kiwada, 2008). Therefore, we also measured the complement activity (CH50) in normal healthy, HS4 day, and HS7 day rats.

As a result, the CH50 in HS4 day and HS7 day rats was significantly decreased compared with that in normal rats [38.0 ± 7.9/ml, 17.1 ± 9.4/ml (p < 0.01), and 30.8 ± 11.0/ml (p < 0.05), for normal, HS4 day, and HS7 day rats, respectively]. However, the degree of the difference between normal healthy and HS7 day rats was remarkably less than that observed between normal healthy and HS4 day rats. These results suggest that the induction of the ABC phenomenon in HS4 day rats is caused by an increase in complement activation, whereas in HS7 day can be mainly attributed to other mechanisms.

**Phagocyte Activity.** Phagocyte activity is strongly related to hepatic uptake and the induction of the ABC phenomenon. Therefore, we hypothesized that phagocyte activity, especially in Kupffer cells, would be altered after resuscitation by HbV injection. To examine the possible changes in phagocyte activity, we estimated the carbon clearance, which is an indication of phagocyte activity in Kupffer cells (Kupffer cells phagocytes were more than 90% of the injected carbon particles).

As shown in the Fig. 4, the phagocyte activity in HS4 day rats was approximately 1.5 times higher than that in normal healthy rats. Of interest, compared with normal healthy rats, phagocyte activity was doubled in the HS7 day rats. These data indicate that phagocyte activity is increased after resuscitation by HbV in the rat model of hemorrhagic shock, and the enhanced phagocyte activity might affect the induction of the ABC phenomenon.

**Discussion**

The induction of the ABC phenomenon can be described for a time frame involving two phases: the induction phase, after the first injection, during which the immune system is primed (reflected in the production of anti-liposome IgM), and the effectuation phase, after the second injection, during which PEG liposomes are rapidly cleared from the bloodstream (reflected in the enhanced uptake by Kupffer cells) (Laverman et al., 2001). In the present study, repeated injections of HbV to a hemorrhagic shock rat model at a dose of 1400 mg Hb/kg seems to induce the ABC phenomenon, and this phenomenon appears to be strongly related to changes that occur during the induction phase, in which the anti-HbV IgM was increased, and the effectuation phase, in which the phagocyte activity in Kupffer cells becomes enhanced by the initially injected HbV.

In the case of the induction phase, it is important to consider the interaction of liposomes with the marginal zone (MZ) in the spleen, which is defined as the junction of the red pulp and white pulp, and contains macrophages, dendritic cells, and B cells (MZ B cells). It was recently proposed that the induction mechanism of anti-liposome IgM involves the localization of liposomes in a certain functional splenic compartment after intravenous injection might be essential and that interaction with immune cells, B cells (but not T cells), in the spleen is critical in the development of this immune response against liposomes (Ishida et al., 2006b, 2007). In addition, it was reported that splenic MZ B cells produce large amounts of IgM within 3 to 4 days after stimulation (Martin et al., 2001). In this study, the production of anti-HbV IgM, but not that of anti-HbV IgG, started from 3 days after the first injection of HbV for resuscitation from hemorrhagic shock (Fig. 1) as well as previous studies using normal rats. Therefore, in the case of HbV injection for a hemorrhagic shock rat model, anti-HbV IgM would be produced via an interaction with splenic MZ B cells, similar to other liposome preparations.

However, it was previously reported that the production of anti-liposome IgM is suppressed with an increase in the first injected dose, and consequently the induction of the ABC phenomenon was inhibited (Wang et al., 2007). Although the dosage amount of HbV in this study was more than 100 times higher than that of other liposome preparations, anti-HbV IgM production was also induced (Fig. 1). This difference can be attributed to differences in physicochemical properties and structure, such as particle size or charge on the surface, between HbV and liposomes used in previous studies. In fact, Demoy et al. (1999) reported that particles with different surface charges and properties and structure, such as particle size or charge on the surface, between HbV and liposomes used in previous studies. In fact, Demoy et al. (1999) reported that particles with different surface charges and diameters showed differences in uptake by the spleen as well as...
localization in the spleen compartment. The diameter and zeta potential of the HbV particles were ~18.7 mV and approximately 250 nm, respectively (Tsushima et al., 2009), whereas the liposomes used in previous reports were ~1.5 mV and approximately 100 nm, respectively (Ishida et al., 2006b). In addition, senescent RBCs are finally captured and degraded by macrophages in splenic MZ cells. Because, unlike other liposomes, the structure of an HbV particle is similar to those of RBCs, it is possible that HbV would interact with MZ cells, which might play an important role in the production of anti-HbV IgM.

The effectuation phase is reflected in an enhanced uptake by the mononuclear phagocyte system, especially Kupffer cells. The carbon clearance measurements showed that systematic phagocyte activity increased by approximately 1.5- and 2-fold at 4 and 7 days, respectively, after the HbV infusion (Fig. 4). A similar phenomenon was recently reported for HbV using normal rats; systematic phagocyte activity began to increase 3 days after an HbV infusion at a dose of 2000 mg Hb/kg, and this value reached a maximum 7 days after HbV infusion (Sakai et al., 2001). From the present limited data, we cannot, with certainty, clarify the mechanism responsible for the enhancement in phagocyte activity that accompanies the administration of HbV. Previous studies reported that the composition of the lipid membrane and the size of the nanoparticles affected the phagocyte activity of mononuclear phagocyte system several days after their infusion in mice (Allen et al., 1984; Fernández-Urrusuno et al., 1996). Therefore, the physicochemical properties of HbV such as the components of the lipid membrane and particle size might also contribute to the induction of phagocyte activity.

Moreover, the possibility that the pathological conditions in our study might have had an effect on the changes in phagocyte activity cannot be excluded. It was previously reported that phagocyte activity, especially Kupffer cells, increased after hemorrhagic shock (Hunt et al., 2001). Under this condition, Kupffer cells exposed to hypoxia and reoxygenation were activated and generated oxidative stress and cytokines, which subsequently further stimulated the Kupffer cells (Rymsa et al., 1991). Moreover, primed and activated Kupffer cells are also stimulated by activated complement factors. Jaeschke et al. (1993) demonstrated that Kupffer cells were activated by complement under conditions of hepatic ischemia reperfusion. In fact, in the previous studies of HbV administration into healthy rats, the complement activation was minimal (Abe et al., 2007; Sou and Tsushima, 2008), and the profile was significantly different from that observed in the present study. Because it is well known that ischemia reperfusion is induced even in the course of hemorrhagic shock and resuscitation, these factors might also be important for the incremental increase in phagocyte activity in this study.

To our knowledge, this is the first examination of the ABC phenomenon using a liposome preparation in conjunction with a model of a pathological condition and provides evidence for the induction of the ABC phenomenon under conditions of hemorrhagic shock. However, our model has several limitations with respect to extrapolating it for use in a human clinical setting. The present studies involved the use of a 40% bleeding model, which was indicated for an RBC transfusion in clinics. Because a massive hemorrhage frequently occurs as the result of a traffic accident or a related injury, it would be expected that the amount of bleeding would exceed 40% of total systemic blood volume. Goins et al. (1995) reported that the circulation kinetics and organ distribution vary among different hypovolemic exchange transfusions with liposome-encapsulated hemoglobin. In addition, the pathological conditions involved, such as blood flow and immunoresponses, can change with the amount of bleeding. Therefore, the induction of anti-HbV IgM and phagocyte activity might be affected by different amounts of bleeding. Similar experiments using a more severe bleeding model should be one of the subjects of future investigation. Moreover, the induction of the ABC phenomenon has been observed in mice, rats, and the rhesus monkey (Dams et al., 2000; Ishida et al., 2003). The injected time interval for the induction of the ABC phenomenon was not consistent with each animal. This implies that extrapolating the present findings obtained using a rat model to human for clinical applications is not an easy task. Therefore, it will be necessary to examine the characteristics of the ABC phenomenon among different animal models of hemorrhagic shock in determining a clinical dosage regimen for HbV.

In conclusion, the present study clearly demonstrates that repeated injections of HbV at a dose of 1400 mg Hb/kg induce the ABC phenomenon in rats under conditions of hemorrhagic shock and that this is associated with the production of anti-HbV IgM and an enhancement in phagocyte activity. These results suggest that, in a clinical situation, the repeated use of HbV in patients with a massive hemorrhage would be expected to induce the ABC phenomenon. Therefore, it may be necessary to considerer the ABC phenomenon in an administration schedule or regimen when HbV is used as a RBC substitute.

Acknowledgments
We thank Emeritus Professor Eishun Tsushima for his support regarding our research.

Authorship Contributions
Participated in research design: Taguchi, Iwao, Watanabe, Kadokawi, and Otagiri.
Conducted experiments: Taguchi.
Contributed new reagents or analytic tools: Sakai, Kobayashi, Horinouchi, and Maruyama.
Performed data analysis: Taguchi and Kadokawi.
Wrote or contributed to the writing of the manuscript: Taguchi, Watanabe, Sakai, Maruyama, and Otagiri.

References

488 TAGUCHI ET AL.