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, Hiroisa 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 125I-HbV, in which the Hb inside of HbV was labeled with 125I. At 4 and 7 days after resuscitation from hemorrhagic shock by nonlabeled HbV (1400 mg Hb/kg), the second dose of 125I-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 (Smuin, 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, Natanson 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).

This work was supported in part by the Ministry of Health, Labor and Welfare of Japan [Health Sciences Research Grants].

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 Hb of HbV was directly labeled with iodine (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 mM pyridoxal 5'-phosphate (Sigma-Aldrich, St. Louis, MO) as an allosteric effector to maintain the HbP50 to 25–28 Torr. The lipid bilayer was a mixture of 1,2-sn-dipalmitoyl-ethanolamine-V-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 maintained in a temperature-controlled room with a 12-h dark/light cycle with ad libitum access to food and water.

Measurement of Carbon Activity (CH50).

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 ultracentrifugation (100,000g, 30 min) to obtain HbV (50,000g, 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-tetrachoro-3a,6a-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 recombinant 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 pentobarbital. Polystyrene 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 = 1/(t2 − t1) × ln(C1/C2), where C1 and C2 are the concentrations (absorbance) at time t1 and t2 (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°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 ± 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 \( ^{125}\text{I}-\text{HbV} \) 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 \( ^{125}\text{I}-\text{HbV} \) 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 \( ^{125}\text{I}-\text{HbV} \) 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 \( ^{125}\text{I}-\text{HbV} \) was also significantly decreased 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 \( ^{125}\text{I}-\text{HbV} \) (percentage of injected dose) at 24 h after \( ^{125}\text{I}-\text{HbV} \) administration. Similar to normal rats, in the HS4 day and HS7 day rats \( ^{125}\text{I}-\text{HbV} \) was mainly distributed in the liver and spleen. However, the amount of \( ^{125}\text{I}-\text{HbV} \) 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

![Figure 1](https://example.com/figure1.png)

**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 resuscitation by HbV at a dose of 1400 mg Hb/kg. After resuscitation, 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 ± S.D. (n = 5).

![Figure 2A](https://example.com/figure2A.png)

**FIG. 2A.** Plasma concentration curve of \( ^{125}\text{I}-\text{HbV} \) after administration to normal (○), HS4 day (□), and HS7 day (■) rats 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 ± S.D. (n = 5). **, *p < 0.01 versus normal rats.

![Figure 2B](https://example.com/figure2B.png)

**FIG. 2B.** Tissue distribution of \( ^{125}\text{I}-\text{HbV} \) (percentage of injected dose) at 24 h after \( ^{125}\text{I}-\text{HbV} \) administration. Similar to normal rats, in the HS4 day and HS7 day rats \( ^{125}\text{I}-\text{HbV} \) was mainly distributed in the liver and spleen. However, the amount of \( ^{125}\text{I}-\text{HbV} \) 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

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>4 day</th>
<th>7 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/2a} ) (h)</td>
<td>5.3 ± 3.9</td>
<td>0.53 ± 0.07*</td>
<td>0.47 ± 0.22*</td>
</tr>
<tr>
<td>( t_{1/2b} ) (h)</td>
<td>30.6 ± 4.0</td>
<td>22.3 ± 3.5*</td>
<td>22.0 ± 3.2*</td>
</tr>
<tr>
<td>( K_e ) (×10³ min⁻¹)</td>
<td>0.70 ± 0.06</td>
<td>1.40 ± 0.38*</td>
<td>1.25 ± 0.18*</td>
</tr>
<tr>
<td>AUC (b % of dose/ml)</td>
<td>210.3 ± 22.9</td>
<td>115.9 ± 24.1*</td>
<td>129.4 ± 12.1*</td>
</tr>
<tr>
<td>CL (ml/h)</td>
<td>0.47 ± 0.04</td>
<td>0.90 ± 0.21*</td>
<td>0.78 ± 0.07*</td>
</tr>
</tbody>
</table>

\( t_{1/2a} \), the distribution-phase half-life; \( t_{1/2b} \), the elimination-phase half-life; AUC, area under the concentration-time curve; CL, clearance.

* *p < 0.01 versus normal.

**TABLE 1**

Pharmacokinetic parameters for HbV after injections of \( ^{125}\text{I}-\text{HbV} \) in normal and hemorrhagic shock model rats

All rats received an injection of \( ^{125}\text{I}-\text{HbV} \) (1400 mg Hb/kg) containing 5% recombinant human serum albumin. At each time after the \( ^{125}\text{I}-\text{HbV} \) injection, blood was collected from the tail vein, and plasma was obtained. Each parameter was calculated by MULTI using the two-compartment model. The values are mean S.D. (n = 5).
Accelerated Blood Clearance of HbV in Hemorrhagic Shock

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 effecuation 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 1,400 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 is increased, and the effecuation 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 diameters showed differences in uptake by the spleen as well as

**Fig. 4.** The phagocyte index (K) in normal (□), HS4 day (■), and HS7 day (■) rats. Carbon clearance was estimated, and K was calculated from the clearance of carbon particles. Each bar represents the mean ± S.D. (n = 5). ***, p < 0.01 versus normal rats.**
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 Tsuchida, 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 to human for clinical applications. Therefore, it may be necessary to consider 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 consider the ABC phenomenon in an administration schedule or regimen when HbV is used as a RBC substitute.
ACCELERATED BLOOD CLEARANCE OF HbV IN HEMORRHAGIC SHOCK


Address correspondence to: Dr. Masaki Otagiri, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp.