DMD Fast Forward. Published on December 1, 2010 as doi:10.1124/dmd.110.036913
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DMD/2010/036913

Repeated injection of high doses of hemoglobin encapsulated liposomes (hemoglobin-vesicles) induces accelerated blood clearance in a hemorrhagic shock rat model

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Running Title: Accelerated blood clearance of HbV in hemorrhagic shock

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The number of text pages: 28

The number of tables: 1

The number of figures: 4

The number of references: 40

The number of words in the *Abstract*: 240

The number of words in the *Introduction*: 718

The number of words in the *Discussion*: 1285

Abbreviations: HBOCs, hemoglobin-based artificial oxygen carriers; **Hb,** hemoglobin; **HbV,** hemoglobin vesicle; **RBC**, red blood cell; **PEG,** polyethylene glycol; **ABC phenomenon,** accelerated blood clearance phenomenon; **^{125}I-HbV,** ^{125}I-labeled hemoglobin vesicle; **MPS,** mononuclear phagocyte system; **MZ**; marginal zone

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 (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 iodine-125. At 4 and 7 days after resuscitation from hemorrhagic shock by non-labeled HbV (1400 mg Hb/kg), the second dose of ^{125}I-HbV (1400 mg Hb/kg) was rapidly cleared from the circulation compared to normal rats. Interestingly, IgM against HbV was produced at 4 days post-first injection of HbV, but decreased at 7 days. In addition, phagocyte activity was increased at both 4 and 7 days post-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 considerer 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 hemoglobin (Hb) (Smani, 2008), have been developed to overcome problems associated with blood transfusion, such as cross-matching, blood-bone infections (human immunodeficiency virus, 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.* recently performed a meta-analysis based on data from randomized controlled trials of 5 different acellular type HBOCs, and concluded that acellular type HBOCs are associated with a significantly increased risk of death and myocardial infarction (Natanson et al., 2008). This 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).

Hemoglobin vesicles (HbVs) are artificial oxygen carriers 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). Since 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; Sakai et al., 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 lifetime in the blood circulation compared to other types of HBOCs (Sou et al., 2005; Taguchi et al., 2009c), guarantees long-term storage for periods of over 2 years at room temperature (Tsuchida et al., 2009). Moreover, HbV possesses oxygen transport characteristics that are comparable to

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; Sakai et al., 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.

It was recently reported that PEGylated liposmes showed some unexpected pharmacokinetic properties, the so-called accelerated blood clearance phenomenon (ABC phenomenon) in which the long-circulation half-life is lost after being administered twice to the same animals (Laverman et al., 2001; Ishida and Kiwada, 2008). Ishida *et al.* recently 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 an accelerated clearance and an enhanced hepatic uptake of the second injected does of PEGylated liposomes (Ishida et al., 2006a). In the case of HbV, there have been several explanation for the induction of the ABC phenomenon as follows; (i) HbV has a liposome structure that contains PEG, (ii) our previous study, using normal mice, showed that the ABC phenomenon was not induced, but anti-HbV IgM was produced 7 days after the post injection of HbV at a dose of 1400 mg Hb/kg (Taguchi et al., 2009c), (iii) the pharmacokinetic properties of HbV are altered under the various pathological conditions (Taguchi et al., 2009a; Taguchi et al., 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 likely 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 ^{125}I-HbV (the internal Hb of HbV was directly labeled with iodine (^{125}I)), 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

HbVs were prepared under sterile conditions as previously reported (Sakai et al., 1997). Briefly, 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 of pyridoxal 5'-phosphate (Sigma Chemical Co., St. Louis, MO) as an allosteric effector to maintain the P_{50} 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 HbVs were suspended in a physiological salt solution at [Hb] 10 g/dL, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with N_2 for storage. The lipopolysaccharide content was <0.1 EU/mL.

Before all experiments, HbV was mixed with recombinant 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-hr 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 removing 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-hr 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 (3000 g, 5 min), and the supernatant was subsequently ultracentrifuged to remove intact HbV (50000 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

^{125}I-HbV was prepared as described in a previous report (Taguchi et al., 2009b). In short, ^{125}I-HbV was prepared by incubating HbV with Na^{125}I (Perkin Elmer, Piscataway, NJ, USA) in an Iodo-Gen (1, 3, 4, 6-tetrachoro-3a, 6a-diphenylglycoluri) tube for 30 min at room temperature. ^{125}I-HbV was then isolated from free ^{125}I by passage through a PD-10 column (Pharmacia Biotech., Uppsala, Sweden). Over 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 (NaI) 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 day (n=5) or 7 day (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 ^{125}I-HbV (1400 mg Hb/kg), blood samples were collected at multiple time points after the ^{125}I-HbV injection (3 min, 10 min, 30 min, 1 hr, 6 hr, 12 hr and 24 hr) and the plasma was separated by centrifugation (3000 g, 5 min). Degraded HbVs and free ^{125}I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% trichloroacetic acid. After collecting the final blood samples (24 hr), the rats were euthanized, and the organs excised (kidney, liver, spleen, lung, heart), rinsed with saline, and weighed. The levels of ^{125}I in the plasma and excised organs were determined using a γ-counter (ARC-5000, Aloka, Tokyo, Japan).

Determination of total blood volume

Total blood volume was determined using the Evans blue dilution technique as previously described, with minor modifications (Kuebler et al., 2004). Briefly, 4 days or 7 days after resuscitation, the rats were 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 formulas: total blood volume = total plasma volume /(100% - hematocrit (%) × (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 health rats without HbV injection (n=5) were also used as controls. In a typical experiment, rats were anesthetized with pentobarbital. Polyethylene 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 Co., Hannover, Germany) was infused at 10 ml/kg within 1 min. At 4, 10, 20, 30, 45 and 60 min later, about 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: $K = 1/(t_2-t_1) \times \ln(C_1/C_2)$, where C_1 and C_2 are the concentrations (absorbance) at time t_1 and t_2 (min), respectively.

Measurement of C activity (CH50)

Ten SD rats were induced with hemorrhagic shock and resuscitated with HbV, and blood samples were collected at 4 or 7 days after resuscitation. The blood was centrifuged (3000 g, 5 min) to obtain plasma for analysis. All plasma samples were stored at -80 °C prior to analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan). The CH 50 was detected by the method of Mayer.

Data analysis

Data are shown as the mean ± SD 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 based on 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 pre-injection 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 that would be used in clinical use (1400 mg Hb/kg) in the rat model of hemorrhagic shock. As shown in Figure 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 a 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 (HS_{4day}) and 7 days (HS_{7day}) after resuscitation by HbV.

Pharmacokinetic study

The fate of the ^{125}I-HbV administered to normal, HS_{4day} and HS_{7day} rats was evaluated by determining residual TCA-precipitable radioactivity in the plasma. Figure 2A shows the time course for the plasma concentration of ^{125}I-HbV in normal, HS_{4day} and HS_{7day} rats and Table 1 lists the pharmacokinetic parameters for these groups. Plasma retention in the HS_{4day} and HS_{7day} rats decreased rapidly compared with that in normal rats, and the plasma clearance (CL) of ^{125}I-HbV in the HS_{4day} and HS_{7day} rats was 1.7 and 1.9 fold increased compared with normal rats (Table 1). Accompanied by a decrease in CL, the area under the time-concentration curve (AUC) was also significantly decreased by half, while the elimination-phase half-life ($t_{1/2\beta}$) of ^{125}I-HbV was also significantly deceased in the hemorrhagic shock model rats compared with normal rats. The pharmacokinetic parameters

DMD Fast Forward. Published on December 1, 2010 as DOI: 10.1124/dmd.110.036913
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were not significantly different between the HS_{4day} and HS_{7day} rats.

Figure 2B shows the tissue distribution of ^{125}I-HbV (% of ID) at 24 hours after ^{125}I-HbV administration. Similar to normal rats, ^{125}I-HbV in the HS_{4day} and HS_{7day} rats was mainly distributed in the liver and spleen. However, the amount of ^{125}I-HbV distribution in the liver was significantly increased in the HS_{4day} and HS_{7day} rats compared to normal rats, while that in the spleen was not significantly different among the three groups. These data indicate that the ABC phenomenon is induced in HS_{4day} and HS_{7day}, 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, HS_{4day} and HS_{7day} rats using Evans blue dilution technique. As shown in Fig.3, the total blood volume in the HS_{4day} was significantly changed, but this change was not remarkable as compared to the massive bleeding. These data indicate that the shorter retention in the circulation in the HS_{4day} and HS_{7day} 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 an 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, HS_{4day} and HS_{7day} rats.

As a result, the CH50 in HS_{4day} and HS_{7day} rats was significantly decreased compared

with that in normal rats (38.0 ± 7.9 /mL, 17.1 ± 9.4 /mL, $p<0.01$, 30.8 ± 11.0 /mL, $p<0.05$, for normal, HS_{4day} and HS_{7day}, respectively). However, the degree of the difference between normal healthy and HS_{7day} rats was remarkably less than that observed between normal healthy and HS_{4day} rats. These results suggest that the induction of the ABC phenomenon in HS_{4day} rats is caused by an increase in complement activation, while that in HS_{7day} 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 phagocyte more than 90 % of the injected carbon particles).

As shown in the Fig.4, the phagocyte activity in HS_{4day} rats was approximately 1.5 times higher than that in normal healthy rats. Interestingly, compared with normal healthy rats, phagocyte activity was doubled in the HS_{7day} 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, following the first injection, during which the immune system is primed [reflected in the production of anti-liposome IgM], and the effectuation phase, following 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 present study, repeated injections of HbV to a hemorrhagic shock rat model at a dose of 1400 mg Hb/kg appears 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 intially 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 following intravenous injection might be essential, and that interaction with immune cells, B cells (but not T cells), in the spleen are critical in the development of this immune response against liposomes (Ishida et al., 2006b; Ishida et al., 2007). In addition, it was reported that splenic MZ-B cells produce large amounts of IgM within 3-4 days after stimulation (Martin et al., 2001). In this study, the production of anti-HbV IgM, but not 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*. reported that particles with different surface charges and diameters showed differences in uptake by the spleen as well as localization in the spleen compartment (Demoy et al., 1999). The diameter and zeta potential of the HbV particles were -18.7 mV and approximately 250 nm, respectively (Tsuchida et al., 2009), while 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. Since, 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 (MPS), 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 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 MPS several days after their infusion in mice (Allen et al., 1984; Fernandez-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 previous 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*. demonstrated that Kupffer cells were activated by complement under conditions of hepatic ischemia reperfusion (Jaeschke et al., 1993). Actually, 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. Since 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. Since 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*. reported that the circulation kinetics and organ distribution vary among different hypovolemic exchange transfusions with liposome encapsulated hemoglobin (Goins et al., 1995). In addition, the pathological conditions involved, such as blood flow and immunoresponces, 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 Prof. Eishun Tsuchida for his support regarding our research. This work was supported, in part, by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan.

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Participated in research design: Taguchi, Iwao, Watanabe, Kadowaki and Otagiri

Conducted experiments: Taguchi

Contributed new reagents or analytic tools: Sakai, Kobayashi, Horinouchi and Maruyama

Performed data analysis: Taguchi and Kadowaki

Wrote or contributed to the writing of the manuscript: Taguchi, Watanabe, Sakai, Maruyama and Otagiri

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Figure Legends

Figure 1

The production of anti-HbV IgG and IgM after resuscitation by HbV in a rat model of hemorrhagic shock.

SD rats were induced by hemorrhagic shock, and resuscitated 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 ELISA. Each bar represents the mean ± SD (n=5).

Figure 2

(A) Plasma concentration curve of ^{125}I-HbV after administration to normal (white circles), HS_{4day} (grey circles) and HS_{7day} (black circles) rats at a dose of 1400 mg Hb/kg.

(B) **Tissue distributions of ^{125}I-HbV at 24 hr after administration to normal (white bars), HS_{4day} (grey bars) and HS_{7day} (black bars) rats at a dose of 1400 mg Hb/kg.**

All rats received ^{125}I-HbV at a dose of 1400 mg Hb/kg, and blood samples were collected at multiple time points (3 min, 10 min, 30 min, 1 hr, 6 hr, 12 hr and 24 hr) and plasma samples were obtained. After collecting final blood sample, each organ was collected at 24 hr after injection. Each point represents the mean ± SD (n=5). **p<0.01 vs. normal rats.

Figure 3

Measurement of total blood volume in normal (white bars), HS_{4day} (grey bars) and HS_{7day} (black bars) rats.

All rats received an intravenous bolus of 1 mg of Evans blue dye in 1 ml of NaCl. After 2 min of distribution time, blood samples (1 ml) were taken; the samples were centrifuged and the absorbance of the samples were measured at 620 and 750 nm. Total blood volume was calculated using the following formulas: total blood volume = total plasma volume /(100% -

hematocrit (%) × (0.01). Each bar represents the mean ± SD (n=5). *p<0.05 vs. normal rats.

Figure 4

The phagocyte index (K) in normal (white bars), HS_{4day} (grey bars) and HS_{7day} (black bars) rats.

Carbon clearance was estimated, and K was calculated from the clearance of carbon particles. Each bar represents the mean ± SD (n=5). **p<0.01 vs. normal rats.

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Table 1

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

All rats received an injection of ^{125}I-HbV (1400 mg Hb/kg) containing 5% rHSA. At each time after the ^{125}I-HbV injection, blood was collected from the tail vein, and plasma was obtained. Each parameter was calculated by MULTI using the two-compartment model.

$t_{1/2\alpha}$, the distribution-phase half-life; $t_{1/2\beta}$, the elimination-phase half-life; AUC, area under the concentration-time curve; CL, clearance

	normal	4 day	7 day
$T_{1/2\alpha}$ (hr)	5.3±3.9	0.53±0.07**	0.47±0.22**
$T_{1/2\beta}$ (hr)	30.6±4.0	22.3±3.5 **	22.0±3.2 **
Ke ($\times 10^3$ min^{-1})	0.70±0.06	1.40±0.38**	1.25±0.18**
AUC (hr % of dose/mL)	210.3±22.9	115.9±24.1**	129.4±12.1**
CL (mL/hr)	0.47±0.04	0.90±0.21**	0.78±0.07**

The values are mean ± SD. (n=5)

**** p<0.01 vs normal**

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Fig.1

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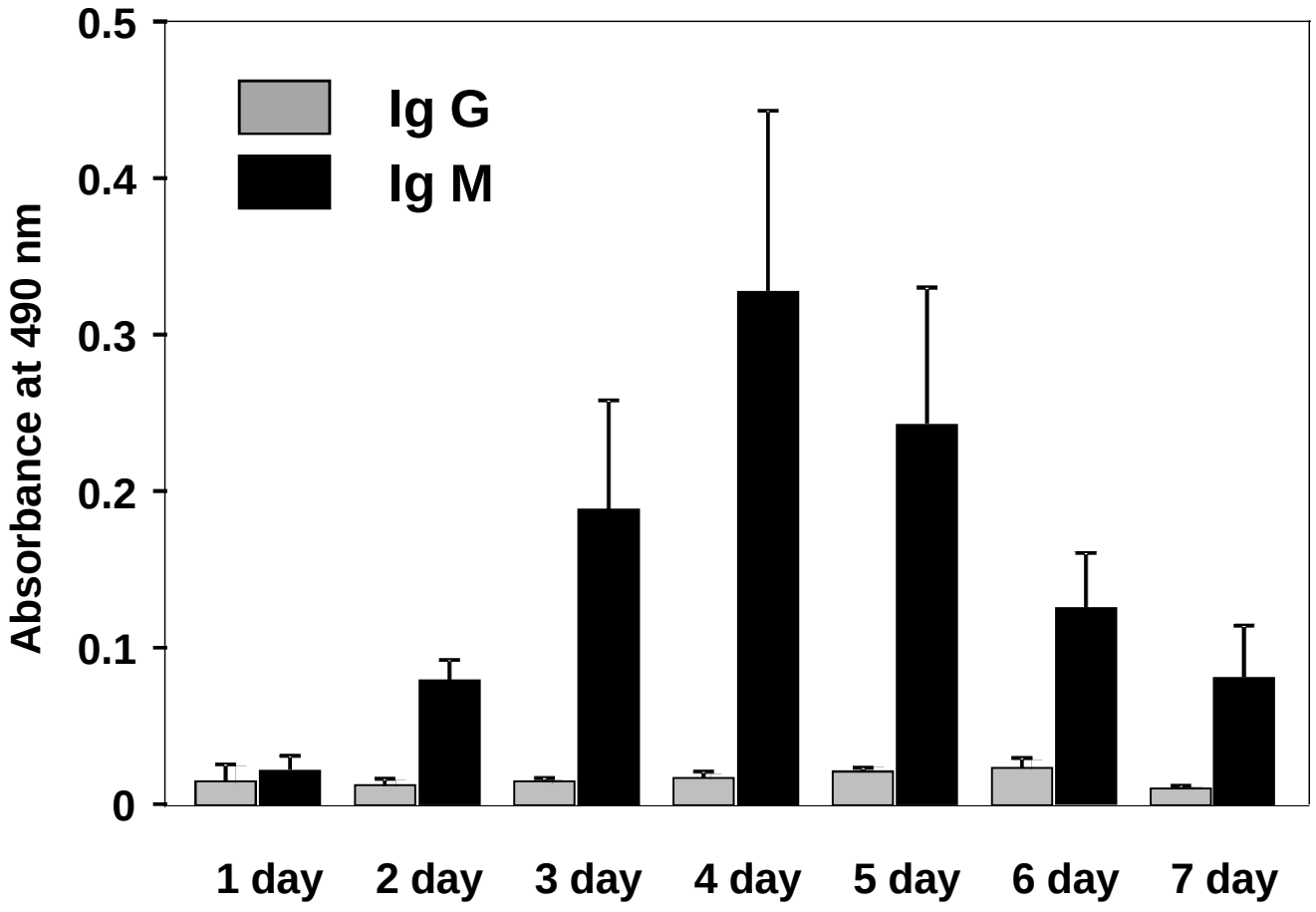

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Fig.2

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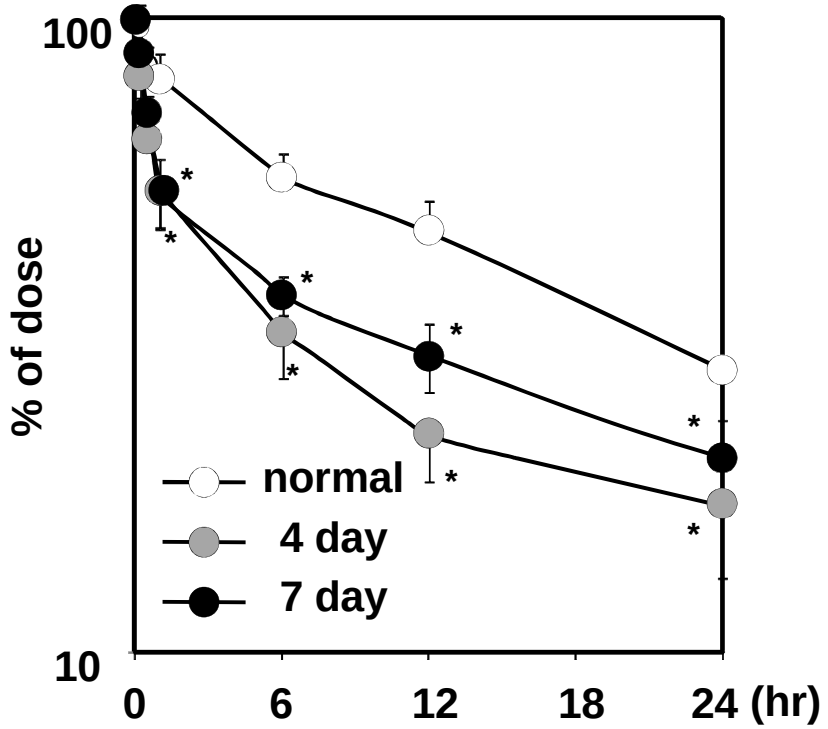
(A)



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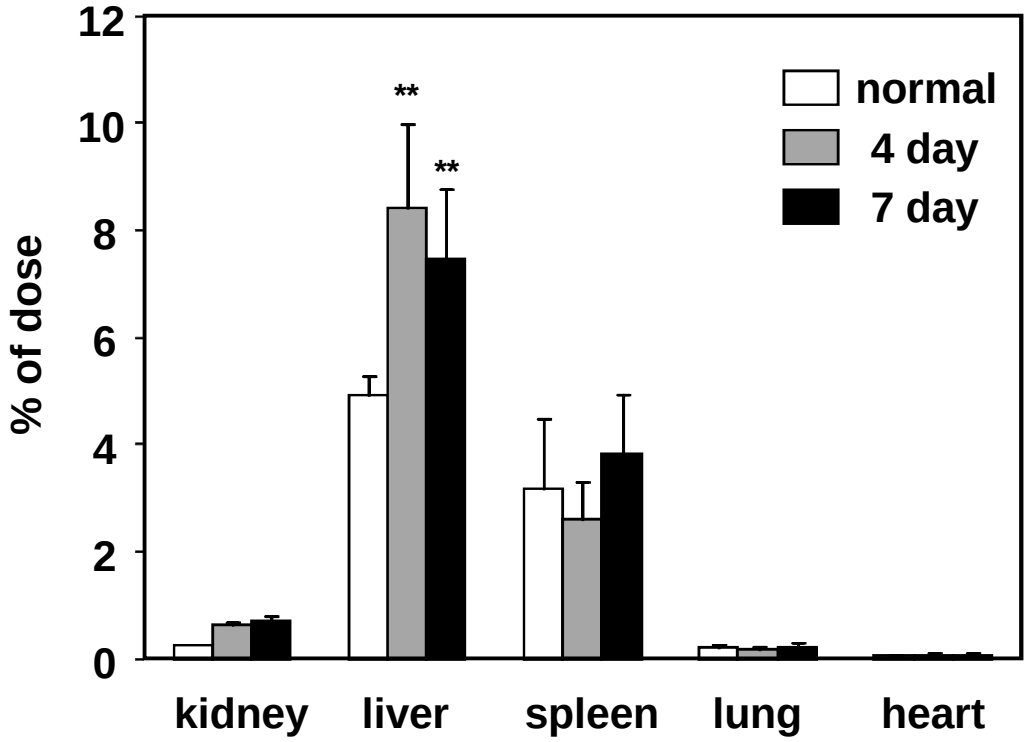

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Fig.3

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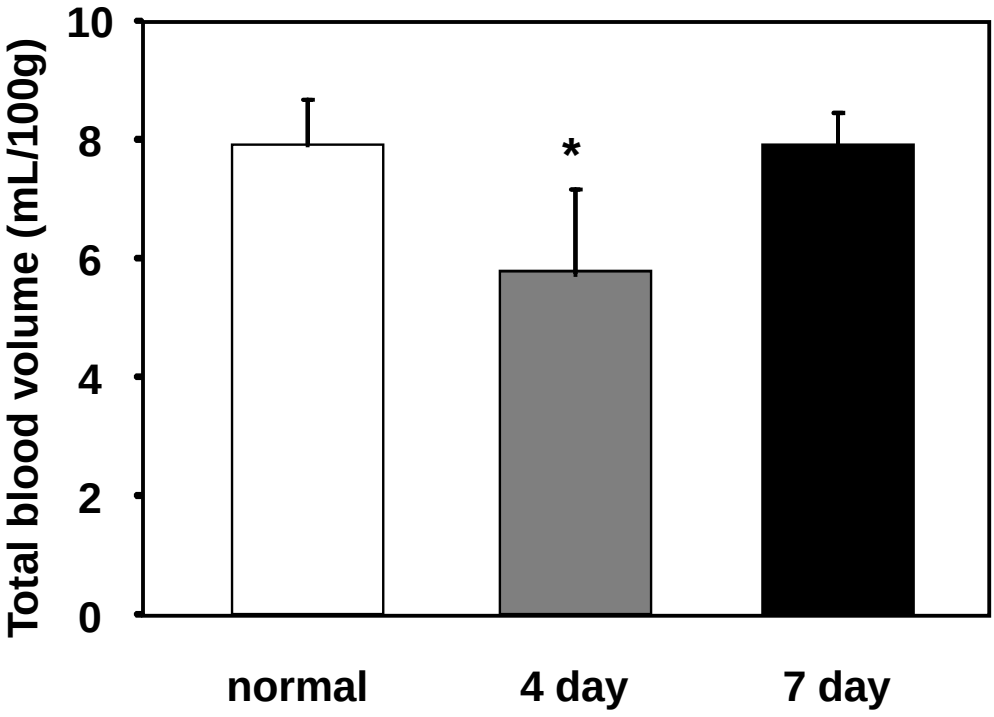

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Fig.4

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