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A Transient Inflammatory Reaction in the Lung After Experimental Hemorrhagic Shock and Resuscitation With a Hemoglobin-Vesicles Solution Compared With Rat RBC Transfusion

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Transfusion for hemorrhagic shock can improve oxygenation, but immunoreactions may induce inflammation. Artificial oxygen carriers have been developed to address clinical concerns of infection and stability, but whether an artificial oxygen carrier might induce inflammation is not well known. To address this question, we compared inflammatory reactions after resuscitation with hemoglobin vesicles (HbVs) or red blood cells (RBCs) in a hemorrhagic shock rat model. Both HbVs and the stored and irradiated rat RBCs deprived of buffy coat were suspended in recombinant human serum albumin [(Hb) = 8.6 g/dL]. Under anesthesia, hemorrhagic shock was induced for 30 min, followed by resuscitation by 20 min transfusion of HbVs or rat RBCs in a volume equivalent to the volume of withdrawn blood. Lungs were excised 2 or 24 h after resuscitation, and mRNA levels of tumor necrosis factor alpha (TNF-α), intercellular adhesion molecule-1 (ICAM-1), nitric oxide synthase 2 (iNOS), nitric oxide synthase 3, hypoxia-inducible factor 1 alpha, and heme oxygenase 1 (HO-1) were measured. In rats resuscitated with HbVs, mRNA levels of TNF-α and HO-1 2 h after resuscitation were significantly higher than those in the rat RBC group, but the levels at 24 h were similar in both groups. The expression of iNOS and ICAM-1, second messengers of inflammation, was not affected, and inflammatory levels after 24 h with HbVs are similar to rat RBC transfusion. The rat RBC group did not show an expected inflammatory reaction related to a transfusion-induced lung injury, and a clinical relevance concerning this level of transient inflammatory reaction induced by HbVs is not known; however, attention to the early stage of resuscitation in ongoing studies of HbV is required. *ASAIO Journal* 2009; 55: 478–483.

Blood transfusion is the primary treatment for massive hemorrhage from injury and during surgery, but various adverse effects have been reported, including transfusion-related acute lung injury (TRALI) and other transfusion reactions, such as graft versus host disease (GVHD), other immunological reactions, and bacterial, viral, and prion infections.[1] To reduce these adverse effects, several types of hemoglobin (Hb)-based artificial oxygen carriers, including liposome-encapsulated Hb, have been developed.[2]

The oxygen carrying capacity of Hb vesicles (HbVs) have been confirmed to be similar to that of red blood cells (RBCs) and are expected to be useful for clinical applications. To prepare these vesicles, purified human Hb is encapsulated with phospholipid bilayer membranes (liposomes), and the vesicular surface is modified with polyethylene glycol chains. HbVs contains no blood group antibodies or infectious pathogens and can be stored for a prolonged period compared to RBCs.[3] In a rabbit hemorrhagic shock model, we have shown that administration of HbVs leads to recovery of hemodynamics and mediates oxygen delivery to vital organs and peripheral tissues to sustain life.[4] In a subsequent study, we compared the hypoxic and inflammatory responses of major organs in a hemorrhagic shock model to the transfusion of either HbVs or Ringer Lactated solution (RL), by quantifying mRNA levels of hypoxia-induced factor 1-alpha (HIF-1α) and tumor necrosis factor alpha (TNF-α). Our results showed that resuscitation from hemorrhagic shock with HbVs did not increase hypoxic or inflammatory effects in major organs, compared with resuscitation using RL solution.[5] However, it is not known whether resuscitation with HbVs after hemorrhagic shock may induce stronger inflammatory reactions than those induced by RBC transfusion. Therefore, we needed to measure the mRNA levels for proteins associated with inflammatory and hypoxic reactions. Tumor necrosis factor alpha (TNF-α) is a cytokine involved in systemic inflammation along with a number of cytokines that stimulate the acute phase reaction.[6] A rapid release of TNF-α plays a central role in the synthesis of adhesion molecules on polymorphonuclear leukocytes (PMNs)[7,8] and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells.[9,10] Furthermore, TNF-α induces nitric oxide synthase 2 (iNOS) and nitric oxide (NO) production in cultured vascular smooth muscle cells.[11,12] NO generated by nitric oxide synthase 3 (eNOS) plays a vital physiological role in maintaining appropriate microvascular tone and blood flow and exhibits protective

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effects.[13] The primary insult of trauma-hemorrhage is the induction of cellular hypoxia. The adaptive response to hypoxia is orchestrated through the induction of HIF-1α.[14] Heme oxygenase 1 (HO-1) also should be examined as an index of an inflammatory reaction and as the index of heme metabolism. By using the indices mentioned previously, we compared inflammatory and hypoxic reactions induced by resuscitation with HbVs and rat RBCs in a hemorrhagic shock model.

Materials and Methods

The study was approved by the Ethics Committee for Animal Experiments at Nippon Medical School. A total of 36 Sprague-Dawley rats aged 10–13 wk and weighing 275 ± 28 g were anesthetized by injection of 30 mg/kg pentobarbital into the tail vein. Anesthesia was maintained with 15 mg/kg pentobarbital during the experiment. Tracheal intubation was performed to allow maintenance of spontaneous respiration under a normal atmosphere. Body temperature was maintained at 36°C–37°C using a hot blanket. Polyethylene catheters were placed in the left femoral artery and vein, through which the mean arterial pressure (MAP) and central venous pressure (CVP) were measured using a transducer (Dinascope DS-3300, Fukuda Denshi, Tokyo, Japan) and arterial blood was sampled.

Blood was withdrawn from the femoral artery over a period of 5 min until a MAP of 30 mm Hg was reached. This level of hypotension was maintained for 30 min by blood withdrawal or by infusion of shed blood. The rats were resuscitated with a 20 min infusion of an HbV/recombinant human serum albumin (rHSA) or rat RBC/rHSA dispersion equivalent to the volume of withdrawn blood. Rats were revived from anesthesia 2 h after transfusion and observed for 24 h after fluid resuscitation. MAP and CVP were measured before hemorrhagic shock (baseline), immediately after shock, immediately after resuscitation, and 1 and 2 h after resuscitation. Arterial blood (0.2 mL) was sampled before hemorrhagic shock (baseline), just before resuscitation, and 1 and 2 h after resuscitation. The arterial blood lactate (Lac) and base excess (BE) levels were measured using an ABL700 instrument (Radiometer A/S, Copenhagen, Denmark). Rats were randomly allocated to sham, HbV, and rat RBC groups (n = 12 per group). Six rats were killed at the 2-h time point, and six rats were killed at the 24-h time point in each group. In the sham group, the duration of anesthesia was the same as that in rats during the induction of shock, but no blood was withdrawn with the exception of blood sampling.

Preparation of HbV and RBC Dispersions

To prepare HbVs for transfusion, a solution of HbVs [Oxygenix Corp., Tokyo, Japan; (Hb) = 10 g/dL, 8.6 mL] was mixed with an rHSA solution (25%, 1.4 mL, Nipro, Osaka, Japan) before use. As a result, the final concentration of the rHSA solution was 5 g/dL. The colloidal osmotic pressure of the dispersion was 20 mm Hg, and the final concentration of HbVs dispersed in rHSA solution was 8.6 g/dL.[15] For the rat RBC transfusion, mannitol-, adenine-, and phosphate (MAP)-supplemented rat RBC concentrate was prepared aseptically following the standard protocol of the Japanese Red Cross Society Central Blood Center for human blood.[16] Blood was collected from donor Sprague-Dawley rats using a heparinated syringe and centrifuged to concentrate the RBCs. Forty milliliters of blood was combined with 6 mL of ACD-A solution (containing 22.0 g/L sodium citrate, 8.0 g/L citric acid, and 22.0 g/L glucose; Kawasumi Laboratories, Japan). After centrifugation of the mixture at 4,000 g for 6 min plasma and the buffy coat were carefully removed, and the precipitate was combined with 9.2 mL of MAP solution (containing 14.57 g/L mannitol, 0.14 g/L adenine, 0.94 g/L sodium dihydrogen phosphate dihydrate, 1.50 g/L sodium citrate, 0.20 g/L citric acid, 7.21 g/L glucose, and 4.97 g/L sodium chloride; Kawasumi Laboratories) to preserve rat RBCs [(Hb) = 10 g/dL]. Then, 1.4 mL of rHSA was added to 8.6 mL of this blood sample and the Hb concentration in rHSA solution was adjusted to 8.6 g/dL, the same condition of the HbV resuscitative fluid. The rat RBC suspension was stored at 4°C for 2 wk and irradiated with γ rays at 15 Gy immediately before transfusion.

RNA Extraction

Animals were killed at appropriate time points, and the lungs were removed. Total mRNA was isolated from the lung using the acid guanidinium-phenol-chloroform method with Isogen solution (Wako Chemical, Tokyo, Japan). The RNA level was determined by measuring the absorbance at 260 nm using a spectrophotometer.

Complementary DNA Synthesis

mRNA was reverse-transcribed using a High Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems Japan, Tokyo, Japan) in a final volume of 20 μL containing 2 μg eluted mRNA, 2 μL 10× reverse transcription buffer, 80 nmol dNTP mixture, 2 μL random primers, 50 U MultiScribe (TM) reverse transcriptase, 20 U ribonuclease inhibitor, and 3.2 μL of nuclease-free water. The reverse transcription reaction was carried out using polymerase chain reaction (PCR) Express (Thermo Fisher Scientific, Waltham, MA) at 25°C for 10 min, 37°C for 10 min, and 85°C for 5 s.

Real Time Reverse Transcription–Polymerase Chain Reaction

The amplification of TNF-α, ICAM-1, iNOS, eNOS, HIF-1α, and HO-1 was performed in a Fast 96-well reaction plate (Applied Biosystems). We used TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan fluorogenic probes. Reactions were carried out in a 20 μL volume containing 10 μL of TaqMan Universal PCR master mix (Applied Biosystems), 1 μL of Taqman probe (Applied Biosystems), 8 μL of deionized water, and 20 ng of cDNA. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to assess DNA integrity. The accession numbers of primers and probes are shown in **Table 1**.

The PCR composed of an initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The Taqman probe labeled with FAM was cleaved during amplification, generating a fluorescent signal and the fluorescence signal was measured after each cycle using the ABI PRISM 7500 Fast Sequence Detector (Applied Biosystems). Results of real-time PCR data were represented as threshold cycle (C_t) values, where C_t represents a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold above baseline. Mark-

Table 1. Code Numbers of Primers and Probes (Applied Biosystems, Foster City, CA)

Gene	Code Number
Tumor necrosis factor alpha (TNF-α)	Rn99999017_m1
Intercellular adhesion molecule-1 (ICAM-1)	Rn00564227_m1
Hypoxia inducible factor 1 alpha (HIF-1α)	Rn00577560_m1
Heme oxygenase 1 (HO-1)	Rn00561387_m1
Nitric oxide synthase 2 (iNOS)	Rn00561646_m1
Nitric oxide synthase 3 (eNOS)	Rn02132634_s1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Rn99999916_s1

edly, different values obtained from triplicate samples, indicating inaccurate operation, were omitted. In practice, if the difference between one C_t value and the average of the other two was >1.00, it was omitted. Relative amounts of all mRNAs were calculated by the comparative C_T method (Applied Biosystems, Foster City, CA).[17,18]

Statistical Analysis

All values obtained from hemodynamic measurements were expressed as means ± standard deviation (SD). Hemodynamic data were analyzed by means of analysis of variance (ANOVA). All values obtained from gene expression measurements were expressed as box and whisker plots showing median, inter-quartile, and full range. Gene expression data were analyzed by Kruskal-Wallis H-test, and a Mann-Whitney *U* test with a Bonferroni correction when a significant difference was found. The significance level was set at $p < 0.05$.

Results

Changes in Hemodynamics

The mean values of blood withdrawal volumes were 9.03 ± 1.54 ml and 9.04 ± 1.74 ml in the HbV group and the rat RBC group, respectively (no significant difference between two groups), indicating that approximately 58% of whole blood was withdrawn under the assumption that the whole blood volume of rats was 56 mL/kg. All the rats that received either HbV or rat RBC survived until the time of organ resection. The hemodynamics during the experiment are shown in **Figure 1**. The baseline values of MAP and CVP were similar in all groups, and MAP and CVP returned to baseline immediately after resuscitation in the HbV and rat RBC groups. No arterial hypertension was observed in both groups. There was also no significant difference in MAP or CVP from baseline at 2 h after resuscitation in any group. The BE level significantly decreased after hemorrhagic shock and increased after resuscitation in the HbV and rat RBC groups, with no significant difference between the groups (**Figure 2**). The Lac level was significantly higher just before resuscitation in the HbV and rat RBC groups compared with baseline and the sham group, but there were no significant differences among the groups at 2 h after resuscitation.

Expression of mRNA

TNF-α mRNA expression was elevated by four times of the sham group 2 h after resuscitation in the HbV group. It was

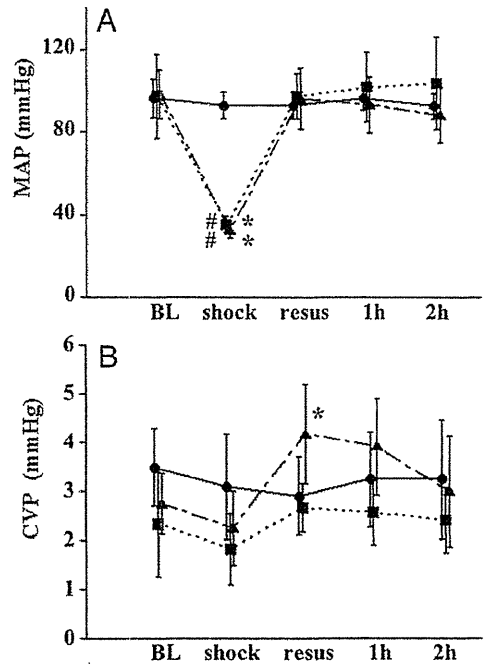

Figure 1. Changes over time in mean arterial pressure (MAP) (**A**) and central venous pressure (CVP) (**B**). BL, baseline; shock, just before resuscitation; resus, just after resuscitation; 1 h, 1 h after resuscitation; 2 h, 2 h after resuscitation. Closed circles: sham group, closed squares: HbV group, closed triangles: rat RBC group. * indicates significant difference from baseline ($p < 0.05$). # indicates significant difference from the sham group ($p < 0.05$).

significantly higher than in the rat RBC group, but there was no significant difference at 24 h (**Figure 3A**). Intercellular adhesion molecule-1 mRNA expression did not differ between the HbV and rat RBC groups throughout the experimental period (**Figure 3B**). iNOS and eNOS mRNA levels after resuscitation showed no significant difference between the HbV and rat RBC groups (**Figure 3C** and **D**). There was a significant difference in HIF-1α mRNA expression between the sham group and both the HbV and rat RBC groups at 2 and 24 h, but no significant difference was found between the HbV and rat RBC groups throughout the experimental period (**Figure 3E**). HO-1 mRNA levels 2 h after resuscitation were greater in the HbV group, but there was no significant difference between the HbV and rat RBC groups 24 h after resuscitation (**Figure 3F**).

Discussion

A series of biochemical and biomechanical changes of RBCs during storage shorten the survival time of these cells and impair function.[19] Moreover, transfusion of blood after storage may increase the risk of multiple organ failure (MOF) development,[20] and complete prevention of infection is very difficult because of unknown infectious diseases and window periods. Use of artificial blood products may overcome these adverse events, and artificial oxygen carriers such as HbVs have been developed as substitutes for RBC transfusion.[3] As shown in **Figures 1** and **2**, the resuscitative effect of HbV was comparable with that of rat RBC transfusion. No arterial hypertension was observed in both groups. Some artificial oxygen carriers scavenge NO and induce vascular constriction

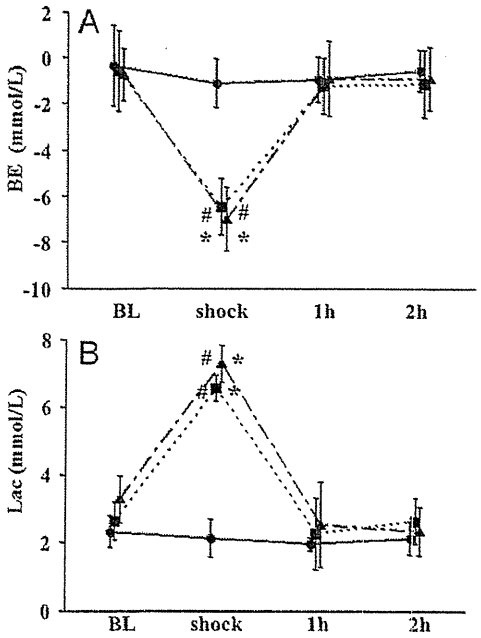

Figure 2. Changes over time in blood base excess (BE) (**A**) and lactate concentration (Lac) (**B**). BL, baseline; shock, just before resuscitation; 1 h, 1 h after resuscitation; 2 h, 2 h after resuscitation. Closed circles: sham group, closed squares: HbV group, closed triangles: rat RBC group. * indicates significant difference from baseline ($p < 0.05$). # indicates significant difference from the sham group ($p < 0.05$).

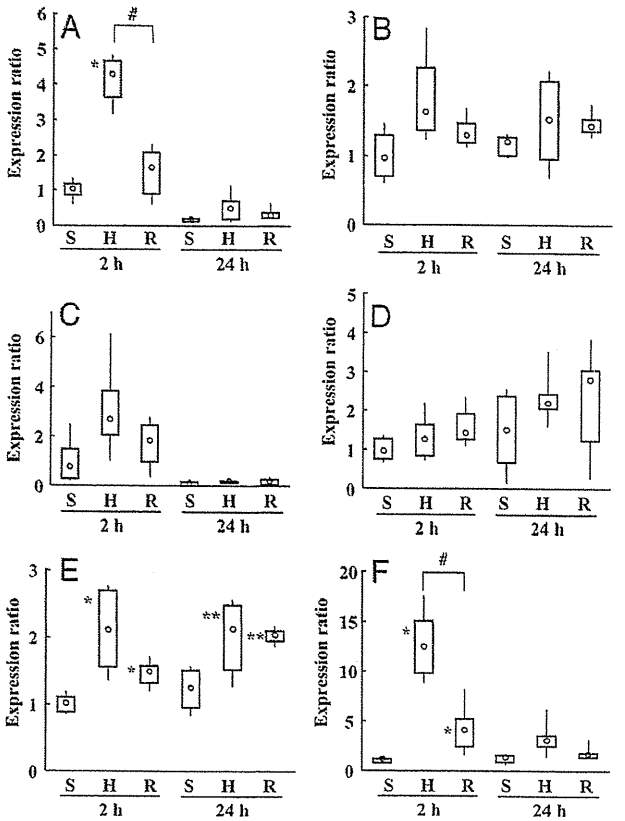

Figure 3. Gene expressions of TNF-α (**A**), ICAM-1 (**B**), iNOS (**C**), eNOS (**D**), HIF1-α (**E**), and HO-1 (**F**) expressed as box and whisker plots showing median, inter-quartile, and full ranges. * indicates significant difference from the sham 2 h group ($p < 0.05$). ** indicates significant difference from the sham 24 h group ($p < 0.05$). # indicates significant difference between HbV and rat RBC group ($p < 0.05$).

and hypertension.[21] In contrast, the reaction of HbV with NO is retarded, and therefore, resuscitation with HbVs did not elevate MAP.[22] Despite such effectiveness of HbV, it was not known how HbV might influence on the inflammatory reactions in the lung after hemorrhagic shock and resuscitation.

It is clinically proven that neutrophils (PMNs)[7,8] and vascular endothelial cells are among the important cellular mediators that induce inflammation after RBC transfusion.[23] Neutrophils, monocytes, and macrophages are thought to be activated by phagocytosis of liposome-encapsulated Hb, with subsequent induction of cytokines.[24,25] According to Ertel *et al.*, the inflammatory reaction after hemorrhagic shock showed significant elevation at around 2 h and began to fall at 24 h. Therefore, we chose similar time points in this study.[26] Our results showed that TNF-α mRNA expression transiently increased immediately after HbV administration, compared with the level of TNF-α mRNA after rat RBC transfusion. However, the mRNA expression levels in the lung at 24 h after resuscitation with HbVs did not differ significantly from those after resuscitation with rat RBCs in the hemorrhagic shock model, showing that inflammatory reactions induced by HbVs were transient and that the conditions after 24 h are comparable to those after rat RBC transfusion. According to the previous studies, acute lung injury (ALI) was induced in rats by hemorrhagic shock followed by resuscitation with shed autologous blood, and the TNF-α mRNA level in the lungs was approximately four times higher than the sham group.[27] TNF-α mRNA levels in lung increased at 3 h and decreased 24 h after resuscitation.[28] Similar to the results in these studies, the expression of an inflammatory cytokine such as TNF-α increased during an early period of inflammation and tended to gradually decrease. In our study, the rat RBC group did not show an expected inflammatory reaction related to a transfusion-induced lung injury, probably because the rat RBCs fraction was carefully deprived of the buffy coat and irradiated with γ rays, and contamination of inflammatory cytokines would be minimized, which might reduce the TNF-α mRNA level in comparison to transfusion of shed autologous blood in the literatures.

In addition to TNF-α mRNA, we measured ICAM-1, iNOS, and HO-1 mRNA levels because they are second messengers of inflammation. Intercellular adhesion molecule-1 is a structural component of the epidermis and endothelial cells, and its expression increases significantly after hemorrhagic shock.[29] In this study, ICAM-1 mRNA levels were not significantly different between the HbV and rat RBC groups. Therefore, the effects of HbVs on the vascular endothelium may be similar to that of rat RBCs, with little effect on inflammation.

NO is an important oxidant radical that is released in lungs and liver by activation of iNOS during hemorrhagic shock.[30] TNF-α plays a role in the induction of iNOS gene expression in fibroblasts, glial cells, cardiomyocytes, and vascular cells.[31] In our study, there were no significant differences in iNOS and ICAM-1 mRNA levels. These results suggest that the transient increase of TNF-α level in the HbV group may not promote the expression of the second messengers, iNOS and ICAM-1.

HO-1 catalyzes the oxidation of heme, carbon monoxide, bilirubin, and iron at an early stage and is the rate-limiting step in these processes.[32,33] HO-1 is induced by a variety of conditions and nonheme substances, such as oxidative stress, heavy metals, endotoxins, heat shock, cytokines, and prostaglandins.[34] Although the HO-1 expression level 2 h after resuscitation was significantly higher after transfusion with HbVs compared with rat RBCs, ICAM-1, and iNOS expression levels were not significantly different. This suggests that HO-1 expression may be enhanced by heme metabolism.

Hypoxia-induced factor-1 α and p38 mitogen-activated protein kinase are associated with fibroblast proliferation and remodeling of the pulmonary artery and rapid hypoxia induced by hemorrhagic shock enhances expression of these molecules in the lung.[35] In this study, the expression level of HIF-1α, a marker of hypoxia, was not significantly different between the HbV and rat RBC groups, although a significant increase was shown after resuscitation for both groups. These results suggest that resuscitation with HbVs is comparable to that with rat RBCs in regard to oxygen transport to tissues.

Conclusion

Our results indicate greater early-stage expression of TNF-α, HO-1 in lungs after resuscitation with HbVs compared with rat RBC transfusion in a hemorrhagic shock model. However, the differences between fluid resuscitation with HbVs and rat RBC transfusion disappeared within 24 h, and the expression of iNOS and ICAM-1 as second messengers of inflammation was not affected in both groups. In our study, the rat RBC group did not show an expected inflammatory reaction related to a transfusion-induced lung injury, and a clinical relevance concerning this level of transient inflammatory reaction induced by HbVs is not clarified. It is necessary to pay attention to the early stage of resuscitation in ongoing studies of HbVs. However, it has to be emphasized that HbVs have an oxygen-carrying capacity equivalent to RBCs in hemorrhagic shock without causing arterial hypertension. We suggest that HbVs is a promising substitute for RBC transfusion.

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5.9　人工酸素運搬体

1．なぜ人工酸素運搬体が必要か

輸血は現代の治療体系の中でも欠くことのできない治療手段であり，救命のために輸血のみが有効である病態（外傷性出血）も多い．しかし，輸血を安全に行うためには，採血，採血時の検査，保存，交差試験，輸血の実施，輸血後の検査といったように多くの人手と時間をかけなければならない．また，採血した血液の保存期間は3週間（欧米では6週間）が限度であり[1]，需給を調整する保存液が改良されたとはいっても保存期間が飛躍的に伸びたわけではない．

輸血が治療として確立してからは，輸血による副作用の解明と克服が輸血を安全に施行するための課題であった．副作用の代表的なものは免疫反応[2]，感染症[3]とGVHD[2]である．免疫反応としては型不適合輸血があるが，major mismatch以外にも非特異抗体による溶血反応もあり，recipientに対してはクームステストなどの検査を追加し，交差試験を行うことが重要となっている．また，最近TRALI（transfusion related lung injury）のような肺傷害が起こることが解明され，このような副作用を防ぐ研究も進められている．感染症については梅毒と肝炎が代表的なものであり，病原体の検索と治療法の開発，汚染血液の排除が重要な命題となっている．梅毒に関してはガラス板法，TPHA試験などにより献血血液から削除することが可能となった．肝炎に関しても病原体の検索が進み，1968年にはHBV（B型肝炎ウイルス），1989年にはHCV（C型肝炎ウイルス）が同定され，汚染血の排除が可能となってきた．HIVは1984年に病原ウイルスが同定されたが，血液製剤の汚染から悲惨な感染を効果的に防ぐことができなかった歴史的背景がある．1999年より核酸増幅法（PCR）を用いた献血血液のNAT検査が全国でもれなく行われるようになって，これらのウイルス感染の機会は非常に低いものとなった．プリオン病の発見によって2002年より欧州滞在者の献血が禁止されたのも記憶に新しい．このようなウイルスなどを克服してきた歴史を考えると，未知のウイルスが献血血液に潜んでいる可能性を排除できず，輸血が治療手段の上で非常に重要な地位を占めるだけに感染症の危険のない血液代替物の開発は現代医学が果たすべき課題の1つである．

また，輸血が行われるためには保存，運搬，交差試験など人がかかわる事務的な部分もあり，ヒューマンエラーをゼロにすることは難しい状況である．

輸血に頼らない治療法の開発（無輸血手術など）も行われているが，限界もある．

以上のような状況から安定した保存が可能で，血液型がなく，いつでもどこでも使用できる輸血代替物の開発が望まれている．

2．人工酸素運搬体研究の歴史

1818年にJames Brundellがヒト-ヒト輸血を成功させたが，危険な治療法であることに変わりはなかった．1900年にヒトに血液型があることがLandsteinerにより発見され，血液型を合わせると輸血が行えることが証明された．1914年にHustinにより，クエン酸ナトリウムによる抗凝固作用が発表され[1]，1915年には冷蔵保存技術が導入されると，輸血は飛躍的に安

全，有効な治療法となり，外科手術も安全な治療手技として種々の手術術式が開発されることとなった．

人工酸素運搬体の開発はこのような輸血治療の確立とともに始まり1920年代にはすでに赤血球よりヘモグロビンを分離して動物やヒトに注入する実験が行われていたようである．第2次世界大戦後はヘモグロビンや金属錯体を用いた酸素運搬体の検討が精力的に行われた．

1966年にClarkとGolanによるパーフルオロ化合物のガス運搬能力が明らかとなり，一躍人工酸素運搬体の開発が進むかと思えた．1981年にはミドリ十字によりパーフルオロ化合物Fruosol DAが開発され[5]，限定的な目的ではあるが，PTCAの際の心筋虚血に対する酸素治療剤として認可を受け，本邦では臨床試験中に大量失血の患者の救命例も報告され，人工酸素運搬体が現実の治療手段であることを印象付けた．Fruosol DAはその後，補体活性や毒性の問題を解消できずに，市場から撤退した．

ヘモグロビンを用いた血液代替物は1988年にChangがコロイジオン球体の中にヘモグロビンを包埋し，酸素の吸脱着を行うことを発表し，これをきっかけに多くの研究がなされるようになった[6]．本邦でもヘモグロビンの分離精製，血液内投与などの基礎的検討からコバルト金属錯体の開発など独自の研究が行われた．

1967年にH Bunnらはヘモグロビン分子間を架橋すると血中滞留時間が飛躍的に延長することを発見し[7]，血液代替物への応用に関する研究が盛んとなり，glutaraldehydeによるHb分子の重合技術（1973），pydoxal 5′ phosphateの分子修飾によるp50の調節技術（1975）などが発表され，80年代に入ると，いろいろな方法による修飾ヘモグロビンの人工酸素運搬体としての開発が盛んとなった．1980年代後半よりBaxterによるdiaspirin crosslinked hemoglobin（DCL Hb）の開発が行われ[8]，phase Ⅲ

表1 修飾ヘモグロビン開発の歴史

修飾Hb（会社名）	成　分	適　応	臨床試験（状況）
PolyHeme™ (Northfield Lab.)	グルタルアルデヒド重合ヒトHb	外傷出血時の輸血代替	第3相（米）開発中止
Hemopure™ (Biopure Co.)	グルタルアルデヒド重合ウシHb	術中輸血代替	第3相（米）pending（南アで認可）
PHP™ (Curacyte)	ピリドキサル化-PEG修飾ヒトHb	敗血症に対する循環動態安定化	第2相（米）
Hemospan™ (Sangart)	PEG-修飾ヒトHb	血漿増量剤	第3相（スウェーデン）
Hemolink™ (Hemosol Co.)	*o*-ラフィノーズ重合ヒトHb	術中輸血代替	第2,3相 pending（米/英/加）
PEG-Hb (Enzon)	PEG-修飾ウシHb	腫瘍組織酸素化	開発中止（米，1997）
HemAssist™ (Baxter)	α-α鎖間分子内架橋ヒトHb	外傷出血時の輸血代替	開発中止（米，1998）（血管収縮）
Optro™ (rHb1.1) (Somatogen)	リコンビナントヒトHb（分子内架橋型）		開発中止（米，1998）（血管収縮）
rHb2.0 (Baxter)	リコンビナントHb（低NO親和度，PEG結合）		開発中止（米，2003）（血管収縮）

の臨床試験を行うまでに開発が進んだ．phase Ⅲ試験は重度外傷における生存率の向上をendpointとして大規模に行われたが，死亡率の増加が認められ，開発が中止された．

また，1980年代には多くのventure businessや，製薬会社による修飾ヘモグロビンの開発が行われた．表1にはその開発の歴史を記した．開発されている製剤は多いが，臨床第3相試験に至った製剤はdiaspirin crosslinked hemoglobin（HemAssist，Baxter社），glutaraldehyde重合ヒトヘモグロビン（PolyHeme，Northfield社），glutaraldehyde重合ウシヘモグロビン（Hemopure，Biopure社），PEG修飾ヒトヘモグロビン（Hemospan，Sangart社）と多くはなく，現在北米ではBiopure，Sangartの各社が開発を継続している（表1）．

セル型の人工酸素運搬体としてはTMS Changの機能評価で酸素運搬が有効であることが示された後に米国Naval Instituteにおいて研究が行われ，技術的な問題がクローズアップされた．早稲田大学では1990年代にヘモグロビン小胞体を開発，テルモ社もNRCの開発名でリポソーム包埋型の人工酸素運搬体の開発研究を行っている．

パーフルオロ化合物の開発においてもFluosol DAの撤退後にパーフルブロン（Alliance社），パーフトラン（ロシア）といった物質の開発が行われ，臨床試験が行われたが，パーフルブロンは脳出血の発生率の上昇などが原因で開発が中止され，企業活動が停止された．しかし，パーフルオロ化合物には液体換気用製剤として先天性横隔膜ヘルニアの呼吸不全に使用して良い成績を上げるなど，人工赤血球としてではない用途での検討も進んでいる．

3．物性としての人工酸素運搬体

人工酸素運搬体が持つべき物性としては，効率的な酸素運搬のために肺で十分に飽和し，末梢組織でかなりの量の酸素を放出する物性を持ち，静脈内に投与して作用し，血中滞留時間が十分にあり，毒性がないことが条件となる．

酸素運搬能が十分という条件を考えると，地球上の物質で，酸素を運搬する可能性のあるものは限られている．生体内で酸素を運搬するタンパクはプロトヘムを含むヘムタンパクであり，代表的なものがヘモグロビンとミオグロビンである．植物まで範囲を広げるとクロロフィル，コルフィセンなども酸素を運搬する．これらの酸素運搬タンパクのうち，血中に投与して酸素運搬体として機能すると思われる分子はヘモグロビンに限定されてしまう．なぜならば，肺胞領域で酸素と結合し，末梢組織に近い毛細血管領域で酸素を解離する性質を持つことが必要で，酸素運搬量が十分大きいことが望ましいからである．ヘモグロビンはαヘモグロビン2分子とβヘモグロビン2分子が会合した特徴的な4量体であり，酸素配位の順位により特徴的なシグモイド型の酸素解離曲線を呈し，大気中の酸素分圧でほぼ100％飽和し，体内の組織における酸素分圧下で40％程度の酸素飽和度となり，多くの酸素分子を効率的に運搬することができる．このため，人工酸素運搬体の開発もこのヘモグロビン分子が主役となっている．その他，物質としては人工合成のヘムを応用した物質（アルブミンヘムなど）や，コルフィセンを修飾したものも検討されている．

人工酸素運搬体は赤血球の酸素運搬能を代替する目的で開発が行われているが，近年では酸素も運搬するplasma expanderとして血圧を保持する輸液製剤としての位置づけで開発されている修飾ヘモグロビンも存在している．

4．人工酸素運搬体の実際

現在開発が進んでいる人工酸素運搬体は大きく分けて，1．修飾ヘモグロビン製剤，2．リポソーム包埋ヘモグロビン，3．パーフルオロ化

合物，4. その他の人工酸素運搬体に分けられる（図1）.

a. 修飾ヘモグロビン

修飾ヘモグロビンは裸のヘモグロビン分子が血中で分離することを防ぐために分子内で架橋を行ったり，ヘモグロビン分子間を重合することにより分子量を増大させる，分子表面を修飾することにより見かけの分子量を大きくする，などの方法で流血中内での滞留時間を延長し，メト化をおさえ，酸素運搬能を保持した製剤が1980年代より開発されている．Baxter社が開発を行ったDCLヘモグロビンは第3相試験で試験群が対照群に比し死亡率が高く毒性もみとめられたため，開発が中止された．この製剤の開発の経緯から，人工酸素運搬体溶液の膠質浸透圧，投与時の血管収縮の副作用，心筋毒性，酸素運搬能について検討が進み，酸素運搬体分子の大きさ，p50の値，重合の方法などを種々変更した物質が開発され，基礎研究，臨床検討が行われた．しかし，多くの製剤が血管収縮の問題点，心筋毒性などを克服できず，臨床応用に至っているものは少ない．現在Biopure社の開発したHemopure（グルタールアルデヒド重合ウシヘモグロビン）が南アフリカで酸素を運搬する血漿増量剤として認可され臨床応用されているのみで，Sangart社のHemospan（PEG修飾ヘモグロビン）は現在臨床試験を行っているところである．本邦でもPLP重合ヘモグロビン（味の素1980年代），PEG-SNO-ヘモグロビン（東北大学-北海道大学グループ）などの開発が行われた．

b. ヘモグロビン小胞体

リポソーム包埋ヘモグロビンは米国海軍の研究グループが研究を行っていたが，リポソームの制御技術の問題から1980年代には開発が中断していた．リポソーム包埋ヘモグロビンの技術的な特徴は，1. リン脂質膜小胞体を安定した脂質二重膜として精製すること，2. Hbの精製，高度濃縮溶液を作成すること，3. 血球成分との相互作用がほとんどない脂質膜修飾法の確立である．たまねぎ状に脂質膜が何層にも重層すると，小胞体内にヘモグロビンを十分に

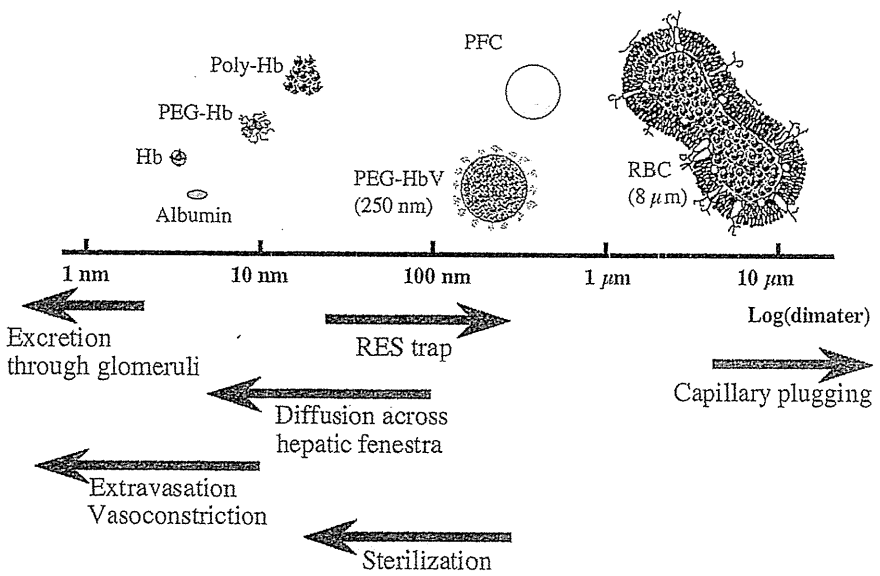

図1 What is the optimal dimension of O_2 carriers?[9)]

包埋できないだけでなく，脂質の投与量が大きくなり，体にかかる負担が大きくなるのと同時に，酸素運搬量が少なくなる．また，小胞体の酸素運搬量を増加させるためには，高度精製されたヘモグロビン溶液を可能な限り高濃度として用いることが必要である．この生成過程で，細菌はもとよりウイルスの不活化も行い，感染症の可能性をほぼ解消することが可能と考えられる．

リポソーム包埋ヘモグロビンによる人工酸素運搬体の開発は1980年代半ばより早稲田大学理工学部高分子研究室で開始され，土田らは高純度に精製されたヘモグロビン溶液（40％）を用いたヘモグロビン小胞体を開発，テルモ社は還元系を残したstroma free hemoglobinを用いた人工酸素運搬体NRCを開発，現在両者は臨床応用へ向けて開発研究が継続中である．

土田らのグループでは上記の技術的課題を1つ1つ解決し，理想的なリポソーム包埋型人工酸素運搬体を作成してきた．脂質二重膜中の負電荷脂質を従来のミリスチン酸から酢酸誘導体に変更し，血小板の活性化がほとんど見られなくなり，PEG修飾により血中半減期が延長し，臨床応用に近づいていると考えられている[9]．

c．パーフルオロ化合物

1970年代にミドリ十字（現田邊三菱）は，パーフルオロデカリンを主体としたフルオゾールDAを開発し，限定目的ながらFDAの承認を受け臨床応用を開始したが，補体活性の上昇と急性毒性のため，大量使用が困難なことが明らかとなり，次第に臨床的価値を失い，市場から撤退した．その後，Alliance社がパーフルブロンを用いた製剤（Oxygent, Liquivent）を開発，臨床試験で良好な結果を得ていたが[10]，血中投与型のOxygentは術中輸血を回避する設定で行った第3相試験において，脳塞栓の増加が明らかとなり開発が中止された[11]．液体換気用に開発されたLiquiventは新生児横隔膜ヘルニアにおける呼吸不全の治療に用いられ，一定の成績を挙げた[12]が，成人の呼吸不全では有効性が証明されなかった．現在開発企業が中国に拠点を移し，輸血代替の面での開発が継続している．

ロシアではPerfutoranと呼ばれるパーフルオロカーボン乳化製剤が開発され，使用されている[13]．

d．その他の人工酸素運搬体

ヘモグロビンタンパクによらないヘムの担体を人工的に作成して血中で酸素を運搬させるというコンセプトにより，種々の物質が開発されてきている．1980年代には早稲田大学理工学部高分子研究室より人工合成のヘムに側鎖をつけてこれを脂質膜小胞体内に埋め込み，40 nmの微粒子とした酸素運搬体を開発，動物試験を行い，ショック蘇生に有効であることが報告されている[14]が，投与直後の血管収縮による血圧の上昇については解明が不十分であった．

また，アルブミンがヘムを包摂する性質を利用して人工合成により安定化したヘム分子をヒトリコンビナントアルブミンに包摂させ，人工酸素運搬体としての機能を付与したアルブミンヘムが1998年に開発された[15]．血中半減期が短いながら，酸素治療薬としての可能性があり，研究の展開に期待がもたれている．

5．人工酸素運搬体の応用分野

人工酸素運搬体開発の最大の目標は輸血代替であるが，輸血に代わるものと考えると，大量・急速の輸注に対する生体反応および安全性に重点が置かれた開発が主体となることは否めない．

輸血代替のほかに酸素運搬体としての特徴を生かした応用がいろいろと考えられている．代表的なものを挙げると，血液型がないことから，体外循環の補填液としての使用，動物用の

輸血代替等が考えられ，運搬体の大きさが赤血球に比し微小であることから，虚血性疾患（心筋梗塞や脳梗塞）の狭窄部を通過あるいは側副血行路よりの病巣部への灌流を考えた梗塞巣の縮小を目的とした使用法，糖尿病による末梢血行障害部位への酸素供給，悪性腫瘍の治療抵抗性を緩和するために腫瘍酸素分圧を上昇させるための酸素治療薬，移植臓器保存時の虚血再灌流障害の緩和を目的とした酸素治療薬，定量的なガスキャリアとしてアイソトープを用いた検査への応用などが考えられており，なかには少量の使用で結果が得られると考えられており，今後の研究展開に期待が集まっている．

6. 見果てぬ夢か―人工酸素運搬体の臨床応用と今後の研究課題

各国で意欲的に進められている人工酸素運搬体の開発であるが，輸血代替の面では最後のブレークスルーが得られていない感がある．1988年より米国FDAは人工酸素運搬体に対する医薬品としての備えるべき物性と生体反応の基準についてたびたび発表を行っており，最近ワシントンDCにおいてヘモグロビンを用いた人工酸素運搬体についてのワークショップを行い，現行の開発方法に対し，疑問と問題点について集中討議を行った．これは，JAMAに発表されたNatansonのメタアナリシス[4]をもとに行われたものであるが，メタアナリシスの対象となった論文がまちまちで，解析の対象として評価するのが適当でないとの批判もあったが，現在開発中の人工酸素運搬体の一般的な副作用の傾向について明らかにし，あらためてFDAの厳しい考え方を示したと考えられた．

今後は，細動脈領域の血管に対する作用や，脳血管および脳実質への影響，心筋毒性の詳細などを解明しつつ，許容できるリスクを明らかにして人工酸素運搬体の臨床応用を図ってゆくことが重要と思われる．

［堀之内宏久，小林紘一，酒井宏水，土田英俊］

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

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ABSTRACT:

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

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

However, Dams et al. (2000) and Ishida et al. (2003a) reported that the intravenous injection of PEGylated liposomes causes a second dose of liposomes to lose their long-circulating characteristics and accumulate extensively in the liver when they are administered twice in the same animal [referred to as the accelerated blood clearance (ABC) phenomenon]. In addition, based on reported liposomal pharmacokinetics data, it is clear that several factors (e.g., size, lipid composition, surface modification, and membrane fluidity) influence the circulating time and the distribution to targeting areas (Ishida et al., 2004; Samad et al., 2007). Ishida et al. (2004) showed that the

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ABBREVIATIONS: PEG, polyethylene glycol; ABC, accelerated blood clearance; HbV, hemoglobin vesicle; Hb, hemoglobin; PLP, pyridoxal 5′-phosphate; MPS, mononuclear phagocyte system; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG; rHSA, recombinant human serum albumin; ^{125}I-HbV, ^{125}I-labeled hemoglobin vesicle; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; CL_{uptake}, uptake clearance; AUC, area under the concentration-time curve; CL, clearance.

physicochemical properties of liposomes, such as lipid composition, diameter, surface modification, and dose, can also have an effect on the ABC phenomenon. Moreover, they also found that anti-PEG IgM, produced by the spleen in response to an injected dose of PEGylated liposomes, is involved in the induction of the ABC phenomenon (Ishida et al., 2006a).

Hemoglobin vesicles (HbV) have been developed as a cellular type of oxygen carrier, in which highly concentrated hemoglobin (Hb) is encapsulated in a phospholipid bilayer membrane with PEG. There are some distinct advantages for HbV to exist in a liposomal structure; the oxygen affinity (P_{50}) of HbV can be easily regulated by manipulating the content of an allosteric effector such as pyridoxal 5′-phosphate (PLP) (Sakai and Tsuchida, 2007). Furthermore, the diameter of HbV liposomes can be tailored to approximately 250 nm, and further modification by PEG leads to an enhanced lifetime in the blood circulation compared with other types of hemoglobin-based oxygen carriers ($t_{1/2}$ for cell-free Hb and PEGylated Hb in rats of 1.5 and 10.9 h, respectively) (Goins et al., 1995; Lee et al., 2006) because the encapsulation of Hb completely suppresses renal excretion, although HbVs in the circulation are eventually captured by phagocytes in the mononuclear phagocyte system (MPS) (Sakai et al., 2001). In fact, our group reported that HbV has a long circulation time in blood as an oxygen carrier in mouse, rat, rabbit, and a hemorrhagic shock model rat (Sou et al., 2005; Taguchi et al., 2009a,b). Because of these unique characteristics, such liposomes show an oxygen transport comparable with red blood cells (Sakai et al., 2008) and also show improved survival in hemorrhagic shock animal models (Sakai et al., 2004b, 2009).

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

In this study, we investigated whether the first injection of HbV at a low dose (0.1 mg Hb/kg), in which the lipid dose induced the ABC phenomenon, as reported by Ishida et al. (2003a), or a high dose (1400 mg Hb/kg), a dose that is proposed for use in a clinical situation, affects the pharmacokinetic behavior of HbV after the second injection in mice. In addition, we also investigated whether anti-HbV IgM is produced after the first injection, and which lipid component of HbV is recognized by IgM.

Materials and Methods

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

Preparation of HbVs. HbVs were prepared under sterile conditions, as previously reported (Sakai et al., 1997). A typical encapsulated Hb (38 g/dl) solution contained 14.7 mM PLP as an allosteric effector to regulate the P_{50} to 25 to 28 Torr. The lipid bilayer comprised a mixture of DPPC, cholesterol, and DHSG at a molar ratio of 5:5:1, and PEG-DSPE (0.3 mol%). The HbVs were suspended in a physiological saline solution at [Hb] 10 g/dl, filter-sterilized (Dismic; Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with N_2 for storage. The content of lipopolysaccharide was <0.1 EU/ml.

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

The Pharmacokinetic Experimental Protocol. All of the animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. All the mice were given water containing 5 mM sodium iodide (NaI) for the duration of the experiment to avoid any specific accumulation in the glandula thyreoidea. Male ddY mice (28–30 g; Japan SLC, Inc., Shizuoka, Japan) were anesthetized using ether and received a single injection of a nonlabeled HbV suspension (0.1 or 1400 mg Hb/kg, 420 μl/30 g) to the tail vein. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received a ^{125}I-HbV suspension to the tail vein under ether anesthesia (the concentration and injected volume were identical to those for the first injection). Each mouse received a total dose of 2×10^6 cpm/30 g ^{125}I activity. At each time after the injection of ^{125}I-HbV, blood was collected from the inferior vena cava under ether anesthesia, and plasma was separated by centrifugation (3000*g*, 5 min). One percent bovine serum albumin (BSA) and 40% trichloroacetic acid were added to the plasma to remove degraded protein and free ^{125}I, and pellets were obtained by centrifugation (1000*g*, 10 min). After collecting blood, the animal was sacrificed for excision of organs (kidney, liver, spleen, heart, and lung), which were rinsed with saline and weighed. ^{125}I radioactivity in the plasma and excised organs was determined using a liquid scintillation counter (ARC-5000; Aloka, Tokyo, Japan).

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

Enzyme-linked immunosorbent assay (ELISA) was used to detect IgG and IgM against HbV using a previously described method, with minor modifications (Wang et al., 2007). The empty vesicles, which contained 475 ng/ml lipids as HbV (comprising DPPC, cholesterol, DHSG, PEG-DSPE at a molar ratio of 5:5:1:0.3) were added to 96-well plates (Immuno 96 MicroWell Plate; Nalge Nunc International, Rochester, NY). The plates were incubated for 2 h at 25°C. After incubation, the wells were washed three times with a wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). A blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was then added to each well, and the plate was incubated for 2 h at 25°C. After incubation, the wells were washed three times with wash solution, and 100 μl of plasma sample, diluted 1:100 with sample solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, 1% BSA, pH 8.0), was added to the wells. After incubation for 90 min, the wells were washed three times with wash solution, and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgG or peroxidase-labeled affinity purified antibody to mouse IgM (μ), diluted 1:1000 with sample solution, was added to each well. After incubation for 60 min, the wells were washed three times with wash solution. Coloration was initiated by adding 100 μl of *o*-phenylene diamine (1 mg/ml). After incubation, the reaction was terminated

by adding 100 μl of 1 N H_2SO_4, and the absorbance was measured at 490 nm using a Microplate reader (model 680; Bio-Rad Laboratories, Tokyo, Japan).

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

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

Results

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

At a dose of 0.1 mg Hb/kg, plasma HbV in the second injection was rapidly cleared compared with that in the first injection (Fig. 1A). The half-life ($t_{1/2}$) in the second injection was reduced significantly—by approximately half—compared with that in the first injection. Accompanied by the reduction in $t_{1/2}$, the area under the concentration-time curve (AUC) was also significantly decreased (27.1 ± 18 and 4.5 ± 3.8 h*% of dose/ml, $p < 0.001$, for first and second injection, respectively), whereas plasma clearance (CL) was significantly increased in the second injection compared with that in the first injection (3.69 ± 0.4 and 22.3 ± 8.1 ml/h, $p < 0.001$, for the first and second injections, respectively). However, the distribution volume of the central compartment (V_1) remained unchanged as the result of repeated injections (Table 1).

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

Effect of Repeated Injection on the Hepatic and Splenic Distribution of HbV. Because liver is the major distribution organ for HbV (Taguchi et al., 2009b), the effect of repeated injections on the hepatic distribution of HbV was examined. Figure 2 shows the time course distribution for ^{125}I-HbV (percentage of injection of dose) in the liver after the administration of ^{125}I-HbV once or twice. Up to 0.5 h after the injection of ^{125}I-HbV at a dose of 0.1 mg Hb/kg, the hepatic distribution of ^{125}I-HbV in the second injection was much higher than that in the first injection (Fig. 2A, inset). However, after 0.5 h or more,

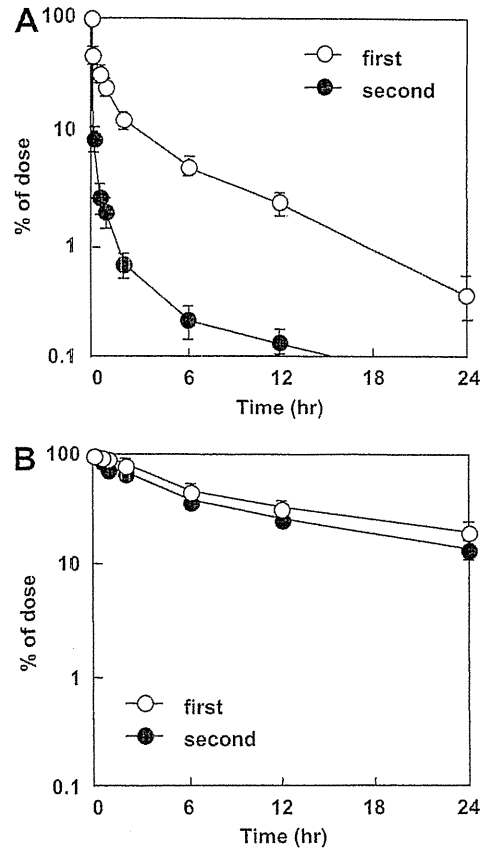

FIG. 1. Plasma concentration curve of ^{125}I-HbV after the first injection (open symbol) or the second injection (filled symbol) of ^{125}I-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of a nonlabeled HbV suspension or ^{125}I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the ^{125}I-HbV suspension to the tail vein. Blood was collected from the inferior vena cava under ether anesthesia, and plasma was obtained. Each point represents the mean ± S.D. (n = 3–6).

the differences between the first and second injections were minor (Fig. 2A). From the beginning after an HbV injection at a dose of 1400 mg Hb/kg, hepatic distributions of ^{125}I-HbV were similar between the first and the second injection (Fig. 2B, inset), and this tendency was maintained for periods of up to 72 h.

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

Because the spleen is an another major distribution organ of HbV (Taguchi et al., 2009b) and an essential organ in terms of inducing the ABC phenomenon (Ishida et al., 2006a), we also examined the time course for the distribution of ^{125}I-HbV (percentage of injection of dose) in the spleen. For periods up to 1 h after HbV injection, the splenic distributions of ^{125}I-HbV in the first and the second injections were not greatly different for doses of both 0.1 and 1400 mg Hb/kg (Fig. 3, A and B, insert). However, 1 h or more after the second injection, higher splenic distributions of HbV were observed in both the low- and high-dose groups compared with those in the first injection (Fig. 3, A and B). In addition, we calculated the CL_{uptake} in

TABLE 1

Pharmacokinetic parameters for HbV after one or two injections of ^{125}I-HbV in mice

Mice received a single or double injection of ^{125}I-HbV (0.1 and 1400 mg Hb/kg) containing 5% rHSA. At each time after the ^{125}I-HbV injection, blood was collected from the inferior vena cava, and plasma was obtained. Each parameter was calculated by MULTI using the two-compartment model. The values are mean ± S.D. (n = 3–6).

	0.1 mg Hb/kg		1400 mg Hb/kg	
	First Injection	Second Injection	First Injection	Second Injection
$t_{1/2}$ (h)	2.7 ± 0.2	1.3 ± 0.3*	18.8 ± 1.3	17.4 ± 3.9
AUC (h*% of dose/ml)	27.1 ± 18	4.5 ± 3.8**	829 ± 38	695 ± 38*
CL (ml/h)	3.69 ± 0.4	22.3 ± 8.1**	0.12 ± 0.04	0.14 ± 0.05
V_1 (ml)	3.1 ± 0.3	3.2 ± 0.3	1.75 ± 0.6	1.81 ± 0.3

V_1, the distribution volume of the central compartment.
* $p < 0.05$, ** $p < 0.001$ vs. first injection.

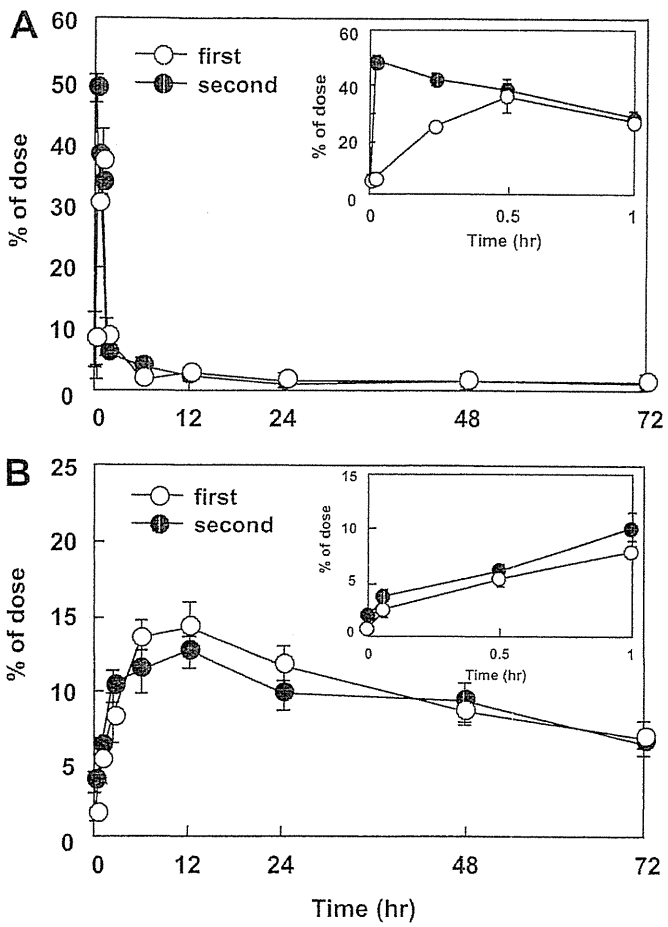

FIG. 2. Time courses for radioactivity in liver after the first injection (open circles) or the second injection (filled circles) of ^{125}I-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or ^{125}I-HbV to the tail vein at a dose of 0.1 or 1400 mg Hb/kg. Seven days after the first injection of the nonlabeled HbV suspension, the same ddY mice received the ^{125}I-HbV suspension to the tail vein. Each point represents the mean ± S.D. (n = 3–6).

the spleen. At a dose of 0.1 mg Hb/kg, the CL_{uptake} for the second injection was 4.5 times higher than that for the first injection (1.6 ± 0.1 and 7.2 ± 3.2 ml/h, $p < 0.01$, for the first and the second injection, respectively). At a dose of 1400 mg Hb/kg, the CL_{uptake} for the second injection (0.07 ± 0.02 ml/h) was not significantly changed compared with that for the first injection (0.05 ± 0.01 ml/h).

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

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

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

Discussion

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

When mice received injections of a low-dose (0.1 mg Hb/kg) HbV, a dose that Ishida et al. (2003b) reported induced the ABC phenom-

TABLE 2

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

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

	0.1 mg Hb/kg		1400 mg Hb/kg	
	First Injection	Second Injection	First Injection	Second Injection
Liver (ml/h)	3.5 ± 0.4	29.6 ± 18*	0.26 ± 0.04	0.37 ± 0.03**
Spleen (ml/h)	1.6 ± 0.1	7.2 ± 3.2*	0.05 ± 0.01	0.07 ± 0.02

* $p < 0.01$ and ** $p < 0.05$ vs. first injection.

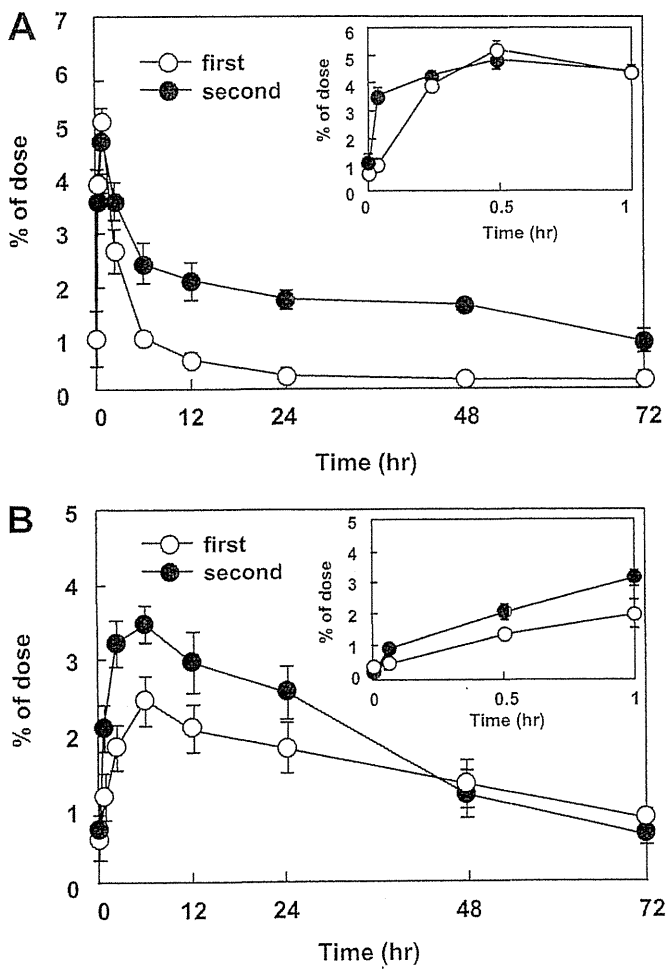

FIG. 3. Time courses for radioactivity in spleen after the first injection (open circles) or the second injection (filled circles) of ^{125}H-HbV to mice at a dose of 0.1 mg Hb/kg (A) or 1400 mg Hb/kg (B). Male ddY mice received a single injection of nonlabeled HbV suspension or ^{125}I-HbV to the tail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

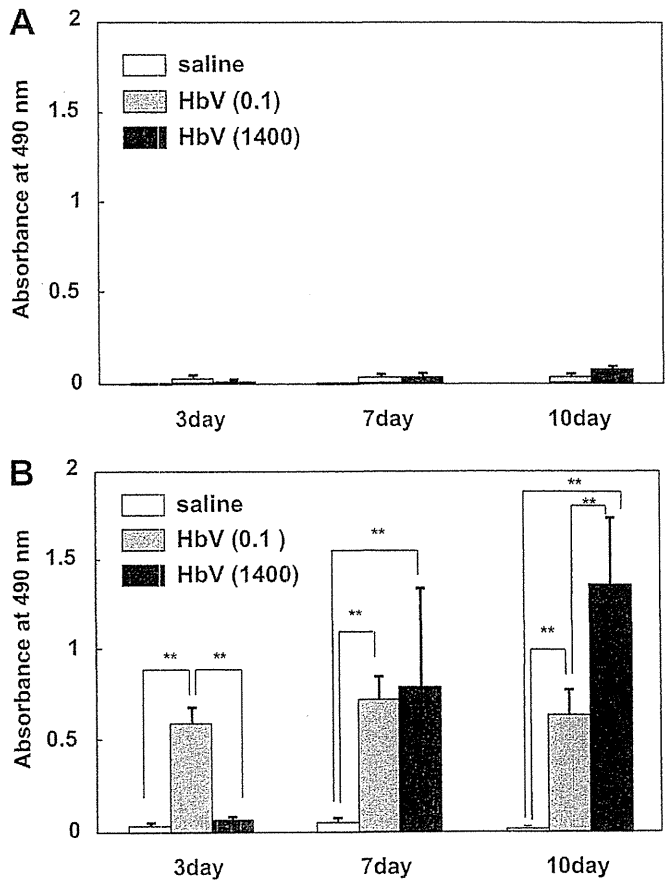

FIG. 4. Determination of IgG (A) and IgM (B) against HbV after a single intravenous injection of saline (open bars), HbV at a dose of 0.1 mg Hb/kg (gray bars) or 1400 mg Hb/kg (closed bars) in mice. The ddY mice received injections of saline or HbV (0.1 or 1400 mg Hb/kg) to the tail vein. At 3, 7, and 10 days after injection of saline or HbV, blood was collected from the inferior vena cava, and plasma was obtained. Anti-HbV IgG and IgM were detected with ELISA. Each bar represents the mean ± S.D. (n = 4). **, $p < 0.01$.

enon, the ABC phenomenon was clearly induced at 7 days postinjection (Fig. 1A; Table 1). Consequently, the pharmacokinetics of HbV was markedly changed. For example, the $t_{1/2}$ and AUC for HbV in the second injection were significantly decreased compared with the values for the first injection, and the CL for the second injection was significantly increased. In addition, the hepatic distribution of ^{125}I-HbV after the second injection at a dose of 0.1 mg Hb/kg was increased for periods of up to 30 min (Fig. 2A) with an increase in hepatic uptake clearance for the second injection (Table 2). In a previous study, Dams et al. (2000) reported that, in mice that were administered liposomes at weekly intervals at a dose of 5 μmol of phospholipids/kg, the ABC phenomenon was not induced. It is well known that a variety of factors, including the lipid dose and physicochemical properties (degree of PEGylation, PEG chain length, surface charge and size) of the initially injected liposome, strongly affect the pharmacokinetic response to subsequent injection (Ishida et al., 2004). For example, it appears that the ABC phenomenon was not caused by preinjection with smaller-sized polymeric micelles but was triggered by preinjection with larger-sized polymeric micelles (Koide et al., 2008). Wang et al. (2005) found that the induction and magnitude of the ABC phenomenon were also influenced by the lipid composition.