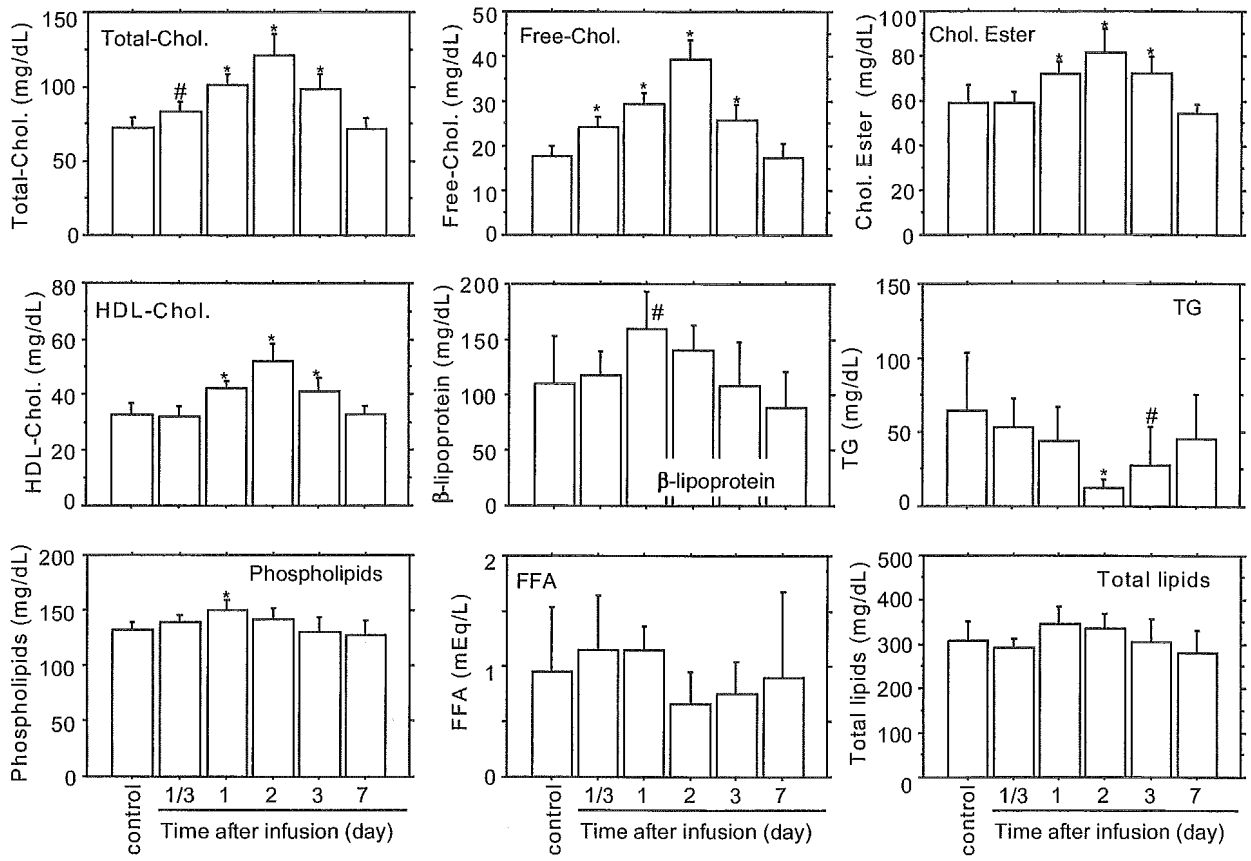

Fig. 4. Plasma laboratory tests representing lipid metabolism after infusion of HbV (20 ml/kg). The values are mean±SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: total cholesterol (Total-Chol.), free cholesterol (Free-Chol.), cholesteryl ester (Chol.Ester), HDL-cholesterol (HDL-Chol.), triglyceride (TG), free fatty acid (FFA).

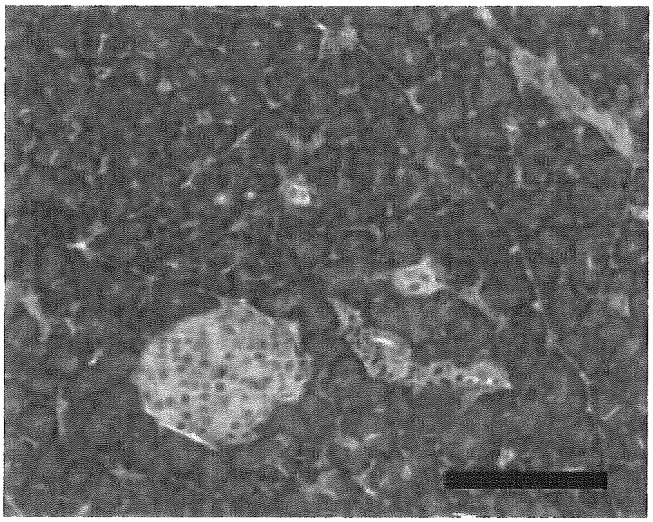

Fig. 5. Histology of pancreas 2 days after the infusion of HbV (20 ml/kg). Bar indicates 100 μm (HE stain).

or metabolized in the same manner. However, a precise pharmacokinetic study is necessary using radiolabeled materials to demonstrate the metabolic and excretory routes of the lipids. Transient, but significant increases in the lipid components raise the necessity of a further study to clarify the influence of a large dose of HbV especially on a lipemic model.

During the metabolism of Hb, there should be a release of bilirubin and iron. However, they did not increase for 7 days. In a previous study, the anti-human Hb antibody staining was effective for detecting the special and temporal distribution of human Hb of HbV both in the spleen and liver [16], and we made it clear that human Hb disappeared within 7 days. The released heme from Hb in HbV may probably be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells in the liver and in the spleen [11,34]. Bilirubin should be excreted in the bile as a normal pathway, and there should be no obstruction or stasis of bile in the biliary tree. Berlin blue staining revealed the presence of hemosiderin 3 and 7 days after HbV infusion, and it disappeared after 14 days [16]. A similar observation was reported for a polymerized Hb that was captured by the Kupffer cells while showing subsequent hemosiderin formation [35]. Normally, iron from a heme is stored in the ferritin molecule [36]. Ferritin in the lysosomal membrane may form paracrystalline structures and

eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Both ferritin and hemosiderin release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation [37,38]. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert [39]. Plasma iron, mostly bound to transferrin, remained constant after HbV infusion. The iron concentration should be coordinately regulated through the "iron regulatory proteins" that sense the levels of iron for hematopoiesis and metabolic needs [40], and the excess amount of iron should be stored in an insoluble and less toxic form as hemosiderin. Together with the time course of the histopathological changes, the results of the plasma laboratory tests indicate that the metabolism of heme and the recycling or excretion of iron molecule is within the physiological capacity and suggested to be on the physiological pathway that has been well characterized for the metabolism of senescent RBC [41].

5. Conclusion

In this study, the plasma laboratory tests after the infusion of HbV (20 ml/kg) did not demonstrate an irreversible sign for a deteriorative damage to the organs. Plasma bilirubin and iron, which were considered to be released during the metabolism of the Hb molecule, did not increase during the observation period. This may be due to the moderate rate of Hb metabolism in RES after the entrapment of HbV with a moderate length of circulation time. The lipid components significantly increased at 2 or 3 days after infusion. These may be derived from the membrane component of HbV entrapped in RES. The complete normalization of the lipid components indicates that they are metabolized in a normal metabolic and/or recycling pathway. The precise biodistribution and fate of the components should be confirmed by a radioisotope technique. Our results have demonstrated the safety of HbV using only healthy rats, while rats in hemorrhagic shock, septic shock, or lipemia have to be tested in the ongoing safety studies. It should also be emphasized that the data cannot be extrapolated to large animals or humans, which may react differently to such a large dose of HbV.

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Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats*

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Objective: **Hemoglobin-vesicle (HbV) has been developed to provide oxygen-carrying ability to plasma expanders. Its ability to restore the systemic condition after hemorrhagic shock was evaluated in anesthetized Wistar rats for 6 hrs after resuscitation. The HbV was suspended in 5 g/dL recombinant human serum albumin (HbV/rHSA) at an Hb concentration of 8.6 g/dL.**

Design: **Prospective, randomized, controlled trial.**

Setting: **Department of Surgery, School of Medicine, Keio University.**

Subjects: **Forty male Wistar rats.**

Interventions: **The rats were anesthetized with 1.5% sevoflurane inhalation throughout the experiment. Polyethylene catheters were introduced through the right jugular vein into the right atrium for infusion and into the right common carotid artery for blood withdrawal and mean arterial pressure monitoring.**

Measurements and Main Results: **Shock was induced by 50% blood withdrawal. The rats showed hypotension (mean arterial pressure = 32 ± 10 mm Hg) and significant metabolic acidosis and hyperventilation. After 15 mins, they received HbV/rHSA, shed autologous blood (SAB), washed homologous red blood cells (wRBC) suspended in rHSA (wRBC/rHSA, [Hb] = 8.6 g/dL), or rHSA alone. The HbV/rHSA group restored mean arterial pressure to 93 ± 8 mm Hg at 1 hr, similar to the SAB group (92 ± 9 mm Hg), which was significantly higher compared with the rHSA (74 ± 9 mm Hg) and wRBC/rHSA (79 ± 8 mm Hg) groups. There was no remarkable difference in the blood gas variables between the resuscitated groups; however, two of eight rats in the rHSA group died before 6 hrs. After 6 hrs, the rHSA group showed significant ischemic changes in the right cerebral hemisphere relating to the ligation of the right carotid artery followed by cannulation, whereas the HbV/rHSA, SAB, and wRBC/rHSA groups showed less changes.**

Conclusions: **HbV suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that using shed autologous blood. (Crit Care Med 2004; 32:539–545)**

Key Words: blood substitutes; artificial red cells; liposome; resuscitation; transfusion

A phospholipid vesicle encapsulating concentrated human hemoglobin (Hb) (Hb-vesicle, HbV) can serve as an oxygen carrier whose oxygen-carrying capacity can be formulated to be comparable to that of blood (1–4). HbV are void of blood-type antigens and infectious viruses and are stable and suitable for long-term storage (5). The cellular structure of HbV (particle diameter, ca. 280 nm) has characteristics similar to those of natural red blood cells (RBCs), because both have lipid bilayer membranes that prevent direct contact of Hb with the components of blood and the endothelial lining. Furthermore, Hb encapsulation in the vesicle suppresses hypertension induced by vasoconstriction, a mechanism presumably due to the effect of free Hb that scavenges the endogenous vasorelaxation factors nitric oxide and carbon monoxide (6, 7) consequent to their high affinity with Hb. Once in the circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (mainly the liver and spleen), and they are metabolized completely within 14 days, with no deposition of iron or lipid (8).

Oxygen-carrying fluids for blood replacement using molecular or encapsulated Hbs have been proposed for volume restoration in hemorrhagic shock (9, 10). We tested the efficacy of HbV suspended in plasma-derived human serum albumin (HSA) in extreme normovolemic hemodilution and found that they are comparable with RBCs (11, 12). In this report, we tested the HbV as a resuscitative fluid for hemorrhagic shock in anesthetized rats. HbV was suspended in recombinant HSA (rHSA), and the efficacy of the resulting HbV/rHSA was compared with that of shed autologous blood and of washed RBCs suspended in rHSA at the same Hb concentration. It has been extensively confirmed that the characteristics of the rHSA are identical with those of conventional plasma-derived HSA

*See also p. 612.

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(13, 14) and that rHSA will soon be approved as a promising plasma expander free from any pathogen from humans.

MATERIALS AND METHODS

Preparation of HbV and Washed RBCs Suspended in rHSA. HbV was prepared under sterile conditions as previously reported (7, 11). Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated purified Hb (38 g/dL) contained 14.7 mM of pyridoxal 5'-phosphate (Sigma Chemical, St. Louis, MO) as an allosteric effector at a molar ratio of pyridoxal 5'-phosphate/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chemical, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF, Tokyo, Japan, 0.3 mol% of the total lipid) (15). HbVs were suspended in a physiologic salt solution at [Hb] = 10 g/dL, sterilized with filters (Dismic, Toyo Roshi, Tokyo, Japan, pore size, 0.45 μm), and deoxygenated with N_2 bubbling for storage (5). The content of lipopolysaccharide was <0.1 EU/mL.

Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25%, 1.4 mL, Nipro, Osaka, Japan) to regulate the rHSA concentration in the suspending medium of the vesicles to 5 g/dL. Under this condition, the colloid osmotic pressure of the suspension is about 20 mm Hg (Wescor 4420 Colloid Osmometer, Wescor, Logan, UT) (11). As a result, the Hb concentration of the suspension was 8.6 g/dL. The viscosities of the suspensions were measured with a capillary rheometer (Oscillatory Capillary Rheometer, OCR-D, Anton Paar GmbH, Graz, Austria). Physicochemical variables of the resulting HbV suspension in comparison with those of the other resuscitative fluids are listed in Table 1.

To prepare washed RBC suspended in rHSA (wRBC/rHSA), blood samples from donor Wistar rats were withdrawn into heparinized syringes and centrifuged to obtain an RBC concentrate. This was washed twice to remove plasma components and buffy coat by resuspension in 5% rHSA and centrifugation (3000 × *g*, 10 mins). The Hb concentration, measured with a cyanometHb method, of the resulting wRBC/rHSA was adjusted to 8.6 g/dL, equivalent to that of HbV/rHSA. The Hb concentration of the shed autologous blood was 13.4 ± 2 g/dL.

Animal Model and Preparation. The experimental protocol was fully approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine. It also complied with the Guide for the Care and Use of Laboratory Animals (16).

Experiments were carried out with 40 male Wistar rats (280 ± 27 g body weight; Saitama Experimental Animals Supply, Kawagoe, Japan). All animals were housed in cages and provided with food and water *ad libitum* in a temperature-controlled room with a 12-hr dark/light cycle. The rats were anesthetized with 1.5%-sevoflurane-mixed air inhalation (Maruishi Pharm., Osaka) with a vaporizer (TK-4 Biomachinery, Kimura Med., Tokyo) throughout the experiment (F_{IO_2} = 21%). Polyethylene catheters (SP-31 tubing, outer diameter 0.8 mm, inner diameter 0.5 mm, Natsume, Tokyo) filled with saline containing 40 IU/mL heparin were introduced through the right jugular vein into the right atrium for infusion and into the right common carotid artery for blood withdrawal. The catheter in the common carotid artery was connected to a Polygraph system (Nippon Koden, Polygraph LEG-1000). The body temperature of the rats was maintained between 37 and 38°C by an isothermal pad (Braintree Scientific, Braintree, MA) during the experiments.

Resuscitation From Hemorrhagic Shock. Hemorrhagic shock was induced by withdrawing 50% of the blood (28 mL/kg, 1 mL/min) from the carotid artery. Systemic blood volume was estimated to be 56 mL/kg body weight (3). Blood was withdrawn into a heparinized syringe and stored for 15 mins at room temperature for the resuscitation with shed autologous blood (SAB). Rats were resuscitated by the infusion of a volume of HbV/rHSA (n = 8), wRBC/rHSA (n = 8), rHSA alone (n = 8), or initially shed autologous blood (n = 8) in 5 min. The volume of the infused resuscitative fluid was identical to the shed volume (i.e., 50% of the blood volume at baseline). To monitor the severity of the shock, eight hemorrhaged rats were not resuscitated with any fluid (nonresuscitated group).

Measurements of Systemic Responses. Systemic variables and blood gases were evaluated before hemorrhage (baseline), after 50% hemorrhage, just after resuscitation, and 1.0, 3.0, and 6.0 hrs after resuscitation. Blood samples were collected in 70 IU/mL heparinized microtubes (125 μL, Clinitubes, Radiometer, Copenhagen) for blood gas analyses and in glass capillaries (Terumo, Tokyo) for hematocrit measurements. A pH/blood gas analyzer (ABL 555, Radiometer, Copenhagen) was used for analysis of Pa_{O_2}, Pa_{CO_2}, pH, base excess (BE), and lactate. A recording system (Polygraph System 1000, Nippon Koden, Tokyo) was used for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). Body temperature was monitored with a thermometer inserted into the anus.

Histopathological Examination and Serum Clinical Laboratory Tests. Six hours after resuscitation, about 5 mL of arterial blood was rapidly withdrawn into heparinized syringes, and the animals were laparotomized and killed by acute bleeding from the abdominal aorta. The liver, spleen, kidney, and then the lung, heart, and brain were resected for a histopathological study. The percentage of the area of ischemic changes (a pyknotic change of nuclei and an edematous change) in the cerebral hemisphere was measured with computer software (IPLab, Fairfax, VA). The blood samples were centrifuged at 3000 × *g* for 5 mins to obtain plasma. The HbV-containing plasma required further ultracentrifugation (50,000 × *g*, 20 mins) to obtain clear plasma avoiding the interference effect of the HbV particles (17). The samples of serum were stored at −80°C before the clinical laboratory tests (BML, Kawagoe). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum activities were measured. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chemical, Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

Data Analysis. Data are given as the mean ± SD for the indicated number of animals. Data were analyzed using analysis of variance followed by Fisher's protected least significant difference test between the groups. The Student's *t*-test was used for the comparisons with baseline values within each group. The level of confidence was placed at 95% for all the experiments.

Table 1. Physicochemical properties of four resuscitative fluids infused into hemorrhagic-shocked rats; hemoglobin-vesicles suspended in recombinant human serum albumin (HbV/rHSA) compared with shed autologous blood(SAB), washed red blood cells suspended in recombinant human serum albumin(wRBC/rHSA), and recombinant human serum albumin (rHSA)

Variables	HbV/rHSA	SAB	wRBC/rHSA	rHSA
Hemoglobin concentration, g/dL	8.6	13.4 ± 2	8.6	0
Particle diameter, nm	281 ± 11	ca. 7000	ca. 7000	—
P_{50}, torr	32	39[a]	39[a]	—
Colloid osmotic pressure, mm Hg	20	22	20	20
Viscosity, cP at 230/sec	2.8	5.2	2.1	1.1

[a]From Reference 12.

RESULTS

Survival Rate. All the rats in the HbV/rHSA, wRBC/rHSA, and SAB groups survived for 6 hrs after resuscitation until the kill. In the rHSA group, two of the eight rats died between 1 and 6 hrs (Fig. 1). Accordingly, hemodynamic and blood-

gas variables (Figs. 2 and 3) of the rHSA group were divided into the survivor group and the nonsurvivor groups. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were six and two, respectively. All the rats in the nonresuscitated group died within 3 hrs.

Systemic Responses to the Hemorrhagic Shock and Resuscitation. MAP of the Wistar rats before hemorrhage was 99 ± 8 mm Hg on the average and declined to 32 ± 6 mm Hg after hemorrhage (Fig. 2*a*). Immediately after resuscitation, the MAP of the SAB group recovered to 110 ± 7 mm Hg, above the baseline value. The value was slightly reduced to 92 ± 9 mm Hg at 1 hr, and the level was maintained for 6 hrs. The MAP of the HbV/rHSA recovered upon retransfusion to 98 ± 8 mm Hg, the baseline level, which was significantly lower than that of the SAB group ($p = .027$). After 1 hr, there was no significant difference between the HbV/rHSA and SAB groups. The HbV/rHSA group showed significantly higher MAP than the rHSA (survivor) ($p = 0.0005$) and wRBC/rHSA ($p = .0032$) groups, whose MAPs at 1 hr were 74 ± 9 and 79 ± 8 mm Hg and remained at this higher level for 6 hrs. The MAP of the nonresuscitated group did not recover and remained at the lowest values. The average HR before hemorrhage was 405 ± 38 beats/min, and there was no significant change after hemorrhage. At 0 hr, the HbV/rHSA ($p = .0215$), SAB ($p = .0085$), and nonresuscitated ($p = .0076$) groups showed slightly lower HR than the basal values; however, there was no noticeable change after that (Fig. 2*b*).

The hematocrit before hemorrhage was 43 ± 2% and was reduced to 36 ± 2% after bleeding due to autotransfusion (Fig. 2*c*). After resuscitation, the hematocrit in the SAB group increased to 42 ± 4%. The hematocrit values in the rHSA (survivor), rHSA (nonsurvivor), and HbV/rHSA groups were significantly reduced to 19 ± 1, 18 ± 1, and 20 ± 2%, respectively ($p < .0001$ vs. baseline), due to the dilution of the blood with the different solutions. The HbV particles remained dispersed in the plasma phase in the glass capillaries for hematocrit measurements. The hematocrit of the wRBC/rHSA group (35 ± 3%) was significantly lower than that of the SAB group ($p < .0001$) corresponding to the lower Hb concentration in the fluid of the wRBC/rHSA groups (8.6 g/dL) than in that of the SAB groups (13.4 ± 2.0 g/dL). The hematocrit of the nonresuscitated group did not change after autotransfusion. The total Hb concentrations in blood after resuscitation with rHSA, HbV/rHSA, wRBC/rHSA, and SAB were estimated to be 6.3, 11, 11, and 13 g/dL, respectively.

Hemorrhagic shock induced metabolic acidosis shown by a decrease in pH from 7.48 ± 0.04 to 7.40 ± 0.09 on the average, a decrease in the BE from 4.5 ± 1.4 to −6.9 ± 3.4 mM, and an increase in lactate from 1.4 ± 0.5 to 6.2 ± 1.4 mM (Fig. 3). As a result, significant compensatory hyperventilation was observed as an increase in Pao_2 of 81 ± 8 torr to 103 ± 6 torr and a decrease in $Paco_2$ of 38 ± 5 torr to 26 ± 5 torr. All the resuscitated groups tended to recover immediately from the hyperventilation after infusion. The pH, BE, and lactate values did not show immediate recoveries after resuscitation but tended to recover at 1 hr. However, they did not return to the baseline level even after 6 hrs ($p < .05$ vs. baseline). There was no significant difference between the HbV/rHSA and SAB group. The nonresuscitated group remained with significant hyperventilation, acidosis, and reduction of BE at 0 hr ($p < .01$ vs. baseline). After that, the Pao_2 decrease and the $Paco_2$ increase were significant in the rats, leading to death. All the variables of the nonresuscitated group were significantly different from those of the HbV/rHSA group at 3 hrs. There was no clear difference between the rHSA (survi-

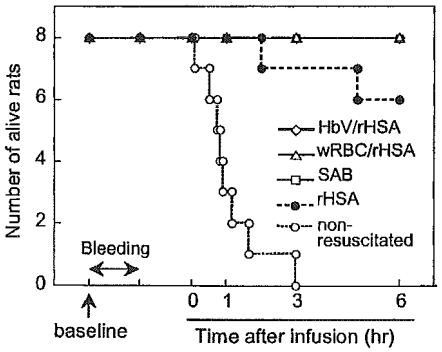

Figure 1. Survival rate of Wistar rats after resuscitation from hemorrhagic shock with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage.

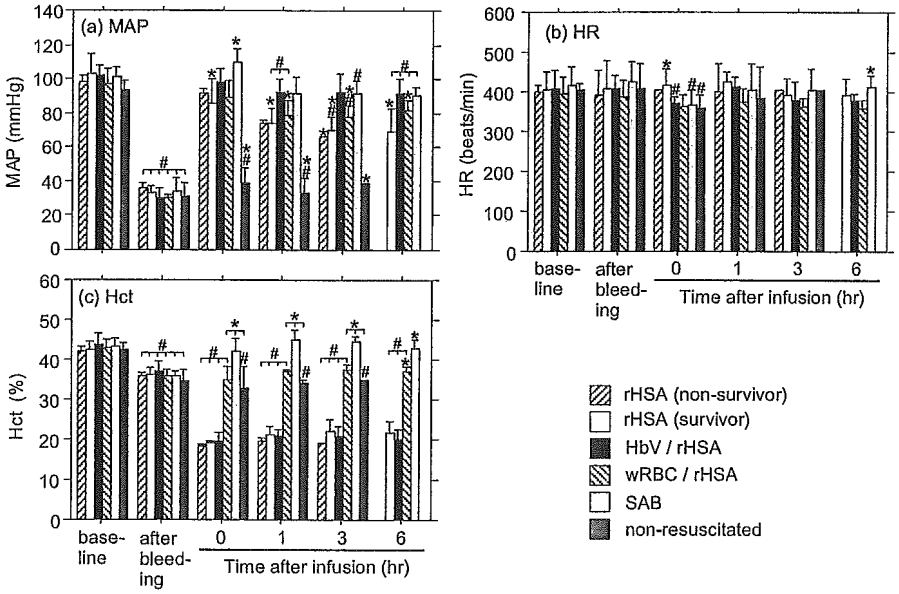

Figure 2. Changes in heart rate (*HR*), mean arterial pressure (*MAP*), and hematocrit (*Hct*) during hemorrhagic shock and resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage and died within 3 hrs (Fig. 1). The number of surviving rats was three at 1 hr. In the rHSA group, two of the eight rats died between 1 and 6 hrs. Accordingly, the rHSA group was divided into the rHSA (survivor) group and the rHSA (nonsurvivor) group until they died. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were 6 and 2, respectively. #Significantly different from baseline ($p < .05$); *significantly different vs. the HbV/rHSA group ($p < .05$).

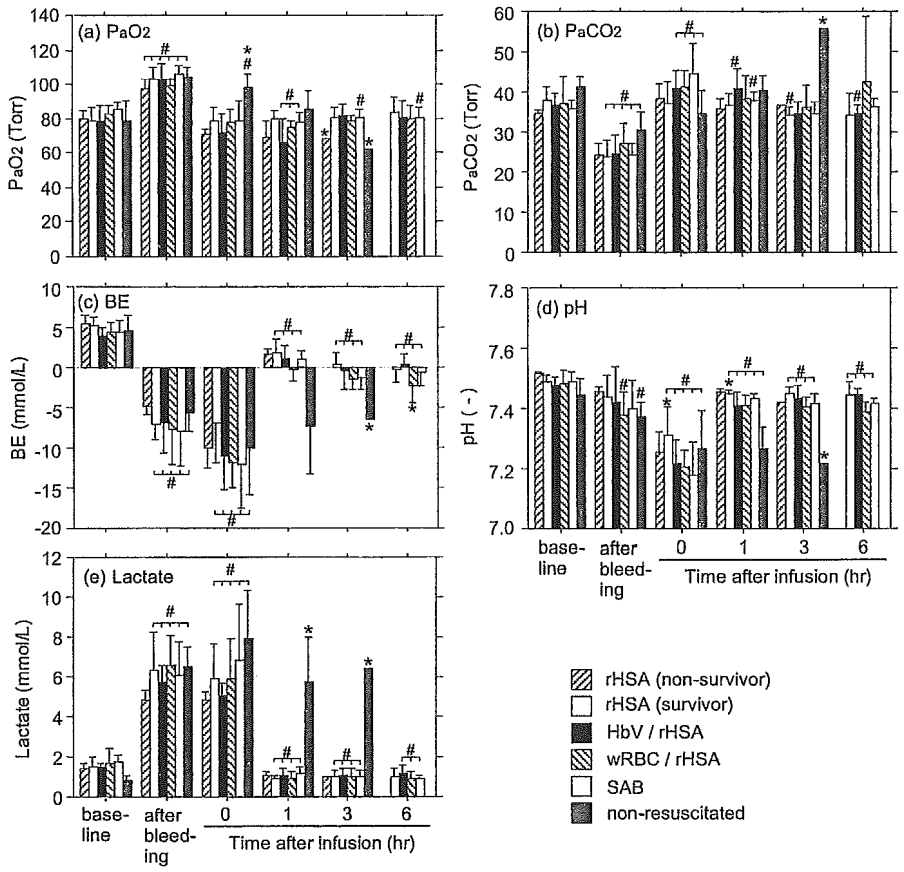

Figure 3. Changes in blood gas variables during hemorrhagic shock and resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. The nonresuscitated group did not receive a resuscitative fluid after the hemorrhage and died within 3 hrs (Fig. 1). The number of surviving rats was three at 1 hr. In the rHSA group, two of the eight rats died between 1 and 6 hrs. Accordingly, the rHSA group was divided into the rHSA (survivor) group and the rHSA (nonsurvivor) group until they died. Therefore, the numbers of rats (n) for the rHSA (survivor) and rHSA (nonsurvivor) groups were 6 and 2, respectively. #Significantly different from baseline ($p < .05$); *significantly different vs. the HbV/rHSA group ($p < .05$). *BE*, base excess.

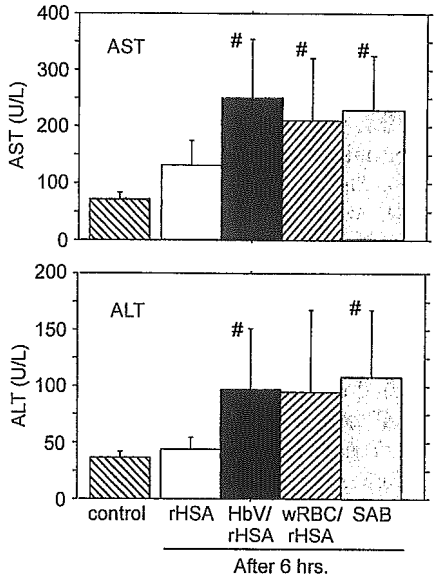

Figure 4. Aspartate aminotransferase (*AST*) and alanine aminotransferase (*ALT*) concentrations 6 hrs after resuscitation with infusion of hemoglobin-vesicles suspended in recombinant human serum albumin (*HbV/rHSA*), shed autologous blood (*SAB*), washed red blood cells suspended in recombinant human serum albumin (*wRBC/rHSA*), and recombinant human serum albumin (*rHSA*) alone. #Significantly different from the control group ($p < .05$).

vor) and rHSA (nonsurvivor) groups in MAP and HR in Figure 2. However, the rHSA (nonsurvivor) group tended to show a slightly lower Pa_{O_2} than the rHSA (survivor) group at 0 and 1 hr and significantly at 3 hrs ($p = .0374$) in Figure 3.

Clinical Laboratory Tests of Blood Serum. Normal Wistar rats showed AST and ALT of 70 ± 13 and 37 ± 5 units/L, respectively (Fig. 4). The HbV/rHSA, wRBC/rHSA, and SAB groups showed significant or nonsignificant increases in AST ($p = .003$, .016, and .005, respectively) and ALT values ($p = .031$, .110, and .025, respectively) compared with the baseline values. On the other hand, the rHSA (survivor) group showed the smallest changes.

Histopathological Examination 6 Hrs After Resuscitation With HbV/rHSA. The hematoxylin/eosin staining of the rat organs demonstrated no significant morphologic abnormalities in the lung, kidney, and liver (data not shown). The red pulp zone of the spleen showed the accumulation of HbV particles as pink-colored dots (8). The myocardium showed focal minimal ischemic changes without apparent necrosis, probably due to the hemorrhagic shock. This histologic finding also was observed in other experimental groups including the rHSA (survivor) group. The cerebral hemisphere on the right side of the rHSA group showed significant ischemic changes, a pyknotic change of the nuclei, and an edematous change (34 ± 3% of the total section area), relating to the ligation of the right carotid artery. However, the other groups that were resuscitated with oxygen-carrying fluids showed minimal changes ($p < .001$ vs. rHSA; HbV/rHSA, 13 ± 5%; SAB, 11 ± 6%; wRBC/rHSA, 11 ± 3%). The nonresuscitated rats that died spontaneously did not show such ischemic changes.

DISCUSSION

One particle of HbV (diameter, ca. 250 nm) contains about 30,000 Hb molecules. HbV acts as a particle in the blood and not as a solute; therefore, the colloid osmotic pressure of the HbV suspension is nearly zero. It requires an addition of a plasma expander for a large substitution of blood such as normovolemic hemodilution to maintain blood volume (18). The candidates of plasma expanders are HSA, hydroxyethyl starch, dextran, or gelatin depending on the clinical setting, cost, countries, and clinicians (19). In this report we tested for the first time the addition of rHSA. The absence of any infectious disease from humans is the greatest advantage of rHSA, which will be soon approved for clinical use in Japan. Moreover, there should be no immunologic and hematologic abnormalities that are often seen with the use of dextran and hydroxyethyl starch (19). The virus inactivation and removal from a human-derived Hb solution can be aggressively

performed in our preparation process of HbV (20, 21). However, to completely avoid unknown infectious diseases, the combination of recombinant Hb-vesicles and recombinant HSA would be the most ideal "artificial red blood cells" in the future.

In our hemorrhagic shock model, all the rats in the nonresuscitated group, which did not receive any fluid, died within 3 hrs, indicating the severity of the shock state. The infusion of the resuscitative fluids resulted in the improvement of all the variables and survival, indicating the importance of the recovery in blood volume. Especially, our principal findings are that the infusion of HbVs suspended in rHSA restores the MAP and blood gas variables including BE and lactate after hemorrhagic shock and that all the rats survived 6 hrs after resuscitation despite the fact that in the rHSA group two rats among eight died within 6 hrs. This clearly shows that the ability of HbV/rHSA as an effective oxygen-carrying resuscitative fluid is comparable with shed autologous blood. After the resuscitation, there were minor differences in blood gas variables between the groups in Figure 3. It would seem that all the animals were hypervolemic in the initial phase of resuscitation, because they experienced autotransfusion during the shock period, although it lasted only 15 mins. This could be one of the reasons there were no significant changes in BE and pH between the groups. Moreover, after the infusion of rHSA, the Hb concentration should not be significantly lower than the transfusion trigger. However, the rHSA group dissociated into the survivor and nonsurvivor groups. There was no remarkable difference between the two, and it was difficult to determine the cause of death. If anything, the rHSA (nonsurvivor) group tended to show the lower Pao_2 values compared with the rHSA (survivor) group ($p = .037$ at 3 hrs), indicating that the respiratory function was not adequate to sustain metabolism under the condition of sevoflurane anesthesia and spontaneous breathing after resuscitation with nonoxygen-carrying fluid. In this case, the combination of the significant hypotension that was seen in all the rats in the rHSA group and the respiratory problem may be one of the causes of incidental death (22). Immediately after resuscitation, the HbV/rHSA group showed a recovery of MAP, Pao_2, and $Paco_2$ that was similar to that of the SAB group. However, the BE, pH, and lactate levels did not show immediate recovery due to the "washing out" of accumulated metabolites including nonvolatile lactate in the peripheral tissues (23). These values recovered 1 hr after resuscitation.

In our previous report (10), we tested HbV suspended in plasma-derived HSA for resuscitation from hemorrhagic shock of conscious small hamsters (ca. 60–70 g body weight) with much lower remaining hematocrit values, maintaining the MAP at 40 mm Hg for 1 hr. Even though the species was different and the observation period after resuscitation was only 1 hr, it seemed that the conscious hamsters showed sufficient compensation of hyperventilation even after resuscitation with HSA alone. In the present study, we could demonstrate the effectiveness of HbV/rHSA in anesthetized rats (280 ± 27 g body weight) as long as 6 hrs after resuscitation, where respiratory function was depressed and compensatory function was not sufficient. This indicates the effectiveness of HbV suspended in a plasma expander for resuscitation from hemorrhagic shock.

It has been reported that resuscitation from hemorrhagic shock with acellular Hb modifications such as polymerized or intramolecularly cross-linked Hb causes the elevation of MAP beyond the baseline values (9, 24, 25), whereas a refined polymerized human Hb that does not contain molecular Hb (<1%) shows no hypertension (26). The hypertension may be presumably due to the high affinity for nitric oxide of molecular Hbs and their smaller size that enables nitric oxide trapping in the proximity of the endothelium (7, 27). However, MAP did not exceed the baseline values after resuscitation with HbV. This is one advantage of cellular HbV in comparison with acellular molecular Hb modifications that may cause vasoconstriction and therefore hypoperfusion of peripheral tissues.

Interestingly, the HbV/rHSA group showed a significantly higher MAP than the wRBC/rHSA group and one that was comparable with that of the SAB group except immediately after resuscitation. It has been extensively confirmed that HbV is not vasoactive and does not induce hypertension (6, 7, 10). Because the total Hb concentrations are identical between the two resuscitative fluids (8.6 g/dL), one of the possible explanations could be related to the more effective oxygen transport by HbV than RBC to the myocardium where the oxygen consumption is significantly large and the oxygen tension gradient is steep. This is speculated from the facts that HbV distributes closer to the endothelial cell layer in the arteriolar blood flow whereas RBCs flow near the axial line (28). Another explanation should be related to the viscosity difference. The viscosity of HbV/rHSA (2.8 cP) is slightly higher than that of wRBC/rHSA (2.1 cP), and this may contribute to the higher vascular resistance and the resulting higher MAP. The slightly higher MAP for the SAB group immediately after infusion may be due to hypervolemia, the trace hemolysis that induces nitric oxide trapping and vasoconstriction, higher viscosity, or clotting during the preservation despite the heparinization.

Histopathological examination of the spleen showed accumulation of HbV in the red pulp zone as previously reported in the study of bolus infusion of HbV in normal rats (8). It was confirmed that HbVs, as foreign particles, were finally captured by the reticuloendothelial system mainly in the spleen and liver, and they were smoothly metabolized within 2 wks. Because the circulation half-life of HbV is about 35 hrs, the spleen had already started to show accumulation of HbV 6 hrs after resuscitation. The lung and kidney did not show any abnormalities such as embolism in the capillaries derived from the aggregation of vesicles (29). In our case, poly(ethylene glycol) modification of the surface of HbV guarantees the homogeneous dispersion and prompt blood flow in microcirculation (11, 30). The complete recovery of the blood gas variables and lactate concentration also supports the normal gas exchanging function of the lung and the excretion and decomposition of metabolites through the kidney and liver, respectively. The myocardium showed a slight influence of ischemic damage for all the groups. The significant difference was observed in the cerebral tissue between the groups receiving oxygen-carrying and noncarrying fluids. The rHSA group showed a significantly larger area with ischemic changes, a pyknotic change of the nuclei, and an edematous change, on the right side; however, other groups receiving oxygen-carrying fluids showed a significantly lower level of changes. We considered that ligation of the right carotid artery and the influence of hypoperfusion induced by the hemorrhagic shock caused ischemic environment in the right cerebral hemisphere, leading to pathologic and irreversible changes of cerebral tissues. The brain tissue was not

Hemoglobin-vesicle suspended in recombinant human serum albumin provides restoration from hemorrhagic shock that is comparable with that using shed autologous blood.

examined in our previous shock study using hamsters with ligation of a carotid artery in the same manner. In the present study, the significantly higher level of ischemic change only in the rHSA group may be caused by the prolonged hypotension and lower oxygen content in blood after resuscitation. Therefore, the cause of death in the rHSA group could be due, in part, to aggravated cerebral damage.

Even though histopathological examination of the liver did not show any abnormalities, the plasma clinical laboratory tests demonstrated elevation of AST and ALT for the HbV/rHSA, wRBC/rHSA, and SAB groups but not for the rHSA (survivor) group. Chemically modified Hbs also were reported to elevate AST and ALT after resuscitation (31, 32). This indicated that the resuscitation with oxygen-carrying fluids might induce ischemia/reperfusion injury that influences liver function (33, 34). However, because AST and ALT values represent the concentration in plasma, the difference in plasma volume between the groups should be considered. The plasma volume ratio should be calculated by subtracting the volumes of RBC and HbV from whole blood. Under the assumption that whole blood volume is equal between the groups, the rHSA group has a 1.35 and 1.20 times larger volume of plasma compared with the SAB group and the HbV/rHSA group, respectively, due to the reduced hematocrit for the rHSA group. Therefore, enzyme concentrations in the rHSA group may possibly be slightly underestimated. Including some antioxidative reagents such as active oxygen scavengers in the resuscitative fluid should be considered to obtain better resuscitation (35, 36).

CONCLUSION

HbV suspended in recombinant HSA at a concentration of only 8.6 g/dL of Hb showed effectiveness for resuscitation from hemorrhagic shock that was comparable to that using shed autologous blood. This acute study encourages us to continue further studies to optimize the physicochemical variables of the HbV suspension such as Hb concentration and oxygen affinity and to look at a longer term survival beyond 6 hrs to weeks using a larger animal model. Some of the polymerized Hbs are now in the final stages of clinical trials (37), and our HbV have to be compared with these materials in terms of safety and efficacy to demonstrate the advantage of cellular structure of HbV.

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Detection of Lipopolysaccharide in Hemoglobin-Vesicles by *Limulus* Amebocyte Lysate Test with Kinetic–Turbidimetric Gel Clotting Analysis and Pretreatment of Surfactant

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ABSTRACT: A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes is necessary because the conventional *Limulus* amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. This interference was evident from isothermal titration calorimetry results in our study that clearly demonstrated the insertion of the LPS molecule into the phospholipid bilayer membrane. Hemoglobin-vesicles (HbVs; particle diameter = 251 ± 80 nm; [Hb] = 10 g/dL) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles, and their oxygen transporting ability has been extensively studied. To accurately measure the LPS content in the HbV suspension, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$), used to release the LPS entrapped in the vesicles, as a pretreatment for the succeeding LAL assay of the kinetic–turbidimetric gel clotting (detecting wavelength, 660 nm). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS at >0.1 endotoxin units (EU)/mL in the HbV suspension. Moreover, the utilization of histidine-immobilized agarose gel effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/mL. Pretreatment with $C_{12}E_{10}$ would be applicable not only to HbVs but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules. J Pharm Sci 93:310–321, 2004

Keywords: liposomes; calorimetry (ITC); surfactants; phospholipids; nanotechnology

INTRODUCTION

Phospholipid vesicles or liposomes have been extensively studied as a drug delivery system since the formation of a vesicular structure was discovered in the suspension of egg yolk phosphatidylcholine,[1] and some are now approved for clinical use as antifungal or anticancer therapies.[2] Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb-vesicles (HbVs) or liposome-encapsulated Hb (LEH), have been developed as oxygen carriers, and their sufficient ability to transport oxygen that is comparable to the ability of blood has been well clarified.[3–7] In comparison with other Hb-based oxygen carriers, such as polymerized Hb or crosslinked Hb, HbVs most closely reproduce the characteristics of natural red blood cells, such as the cell membrane function of physically preventing the direct contact of Hb

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with the components of blood and vasculature during circulation.[8–11] The advantages of artificial oxygen carriers are the absence of blood-type antigens and infectious viruses and stability for long-term storage.[12] In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion, which still has the potential of mismatching, the risk of infections secondary to infusion of contaminated blood, and the problem of only a few weeks storage life.[13]

The process of production of a HbV has to be guaranteed with a good manufacturing practice (GMP) standard so that the HbV is a biological product that adheres to the strict regulations of impurity and viral and bacterial contamination. It is strictly required to monitor the content of the lipopolysaccharide (LPS), known as an endotoxin, which is a component of the outer membrane of gram-negative bacteria that possesses a large variety of biological influences on numerous mammalian cells and tissues.[14] An endotoxin is an extremely potent toxin with lethal doses (LD_{50}) of 3 and 1 mg/kg in rats and dogs, respectively.[15,16] The U.S. Food and Drug Administration (FDA) has established a guideline for human maximal permissible endotoxin dose for parenteral products [5 endotoxin units (EU)/kg][17] that may include Hb-based oxygen carriers. This limit is based on the endotoxin activity (1 EU = 100 pg) and can be measured by the *Limulus* amebocyte lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS.[18] In general, the LAL method has advantages over the rabbit pyrogen testing because the LAL method requires a lower amount of sample and the assays can easily be repeated.[19] Because the volume of oxygen carriers to be infused for shock resuscitation or acute hemodilution is estimated to be <20 mL/kg, the specific endotoxin limits should be 0.25 EU/mL (= 5/20), which is similar to that for water for injection (0.25 EU/mL).

Bacterial LPS is an amphiphilic gigantic macromolecule, therefore, it hydrophobically interacts with protein and biomembranes.[20] Hb strongly interacts with LPS, showing synergistic toxicity.[21–23] The constituent of endotoxin that causes LAL gelation is a glycophospholipid that is designated lipid-A.[24] Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A, such as its LAL gelation activity.[25,26] As a consequence, the researchers who study HbVs or other phospholipid vesicles for delivering other functional molecules encountered a problem in measuring the LPS content for the quality control of these materials.[27–30] Considering this background information, we aimed to find the optimal condition for the pretreatment of HbVs using a surfactant to release LPS[28,29] with a minimal interference effect for the subsequent kinetic–turbidimetric LAL assay using a Toxinometer®. For a better detection limit, we tested the histidine-immobilized agarose gel that effectively adsorbs LPS to concentrate the trace amount of LPS and to eliminate the solutes that interfere with the LAL assay.[30–33]

EXPERIMENTAL

Preparation of Poly(ethylene glycol) (PEG)-Modified Hb-Vesicles (HbVs)

The PEG-modified HbVs were prepared under sterile conditions as previously reported.[8,34–36] Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and Japanese Red Cross (Tokyo, Japan). The purification process included ultrafiltration to remove the stromal components and pasteurization at 60°C for 10 h to denature the concomitant proteins. This process results in extremely high purity of Hb (>99.9%).[35,37] The encapsulated Hb (38 g/dL) contained 14.7 mM pyridoxal 5′-phosphate (PLP, Merck Company, Darmsdart, Germany) as an allosteric effector at a PLP/Hb molar ratio of 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (DPEA) at a molar ratio of 5:5:1 (Nippon Fine Chemical Company, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (PEG–DSPE; NOF Company, Tokyo, Japan).[38] Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DPEA/PEG–DSPE was 5:5:1:0.033. The HbV particles were suspended in saline at an Hb concentration of 10 g/dL. The physicochemical parameters of the HbV were as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dL; [metHb], <3%; [carbonylhemoglobin (HbCO)], <2%; lipids, 5.6 g/dL; and oxygen affinity (P_{50}), 31 Torr. All the analytical methods are described elsewhere.[12]

Thermodynamic Analysis of Insertion of LPS into Vesicles by Isothermal Titration Calorimetry (ITC)

An OMEGA titration microcalorimeter (MCS ITC, Microcal Inc., Northampton, MA) was used to analyze the interaction of LPS with the phospholipid bilayer membrane.[38,39] The model phospholipid vesicles of DPPC/cholesterol/DPEA/PEG–DSPE (5:5:1:0.033 by mol) were prepared under sterile conditions by simply dispersing the lipid powders in pure water (0.05 g/dL). The resulting suspension was freeze-thawed to enhance the dispersion and to regulate the particle diameter to 519 ± 78 nm. Twenty-five cumulative injections of an LPS solution (10 μL, 334 μM in pure water, from *Salmonella Minnesota* wild type, MW = ~20 kDa[40]; Sigma Chemical Company, St. Louis, MO) into the vesicle suspension (1.35 mL, [lipid] = 835 μM) were performed using a computer-controlled microsyringe while the suspension was stirred at 400 rpm and the temperature was strictly controlled at 37°C. Changes in the calorific values and the total calorific values were automatically measured. An identical injection of LPS into pure water in the absence of vesicles and injection of pure water into the vesicle suspension were performed as references.

Determination of the Solubilization Condition of HbV with Surfactants

An HbV suspension, diluted twice or five times with pure water ([Hb] = 2 or 5 g/dL, 200 μL), and deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$, 0–10 vol% dissolved in pure water, 800 μL; Sigma Chemical Company) were mixed in a quartz cuvette (2 mm thickness). The resulting suspension was heated at 42°C for 2 min to enhance the solubilization. The turbidity was measured at 660 nm with an ultraviolet–visible (UV–vis) spectrophotometer (V-560, Jasco, Tokyo). For comparison, other surfactants that are often used for the dissolution of liposomes or biomembranes,[28,29,41] [sodium dodecyl sulfate (SDS), Kanto Chemical Company, Tokyo, Japan; Triton-X100 and nona(oxyethylene) dodecyl ether ($C_{12}E_9$), Sigma Chemical Company] were also tested for the solubilization of HbV.

Calibration Curves for LAL Assay and Influence of a Surfactant

A powdered LPS (LPS purified from *Escherichia coli* UKT-B containing mannitol and glycine, Wako Pure Chemical Industries, Tokyo, Japan), a control standard LPS defined in Japanese Pharmacopoeia, was dissolved in pure water (Water for Injection, Otsuka Pharmaceutical Company, Tokyo, Japan) to prepare the stock solution ([LPS] = 500 EU/mL). The LPS standard solutions ([LPS] = 0.01~20 EU/mL) were prepared by dilution of the stock solution with pure water. The LPS solutions (200 μL) were mixed with a $C_{12}E_{10}$ solution (0–5 vol%, 800 μL), diluted eight times with pure water, and then mixed with an equal amount of LAL solution (*Limulus* ES-II Test Wako, Wako Pure Chemical Industries) containing a lysate from *Limulus polyphemus*, Tris-HCl buffer (pH = 7.1), and a derivative of β-1,3-glucan. The LPS content in the water for injection is below the detection limit (<0.0001 EU/mL). The LPS content was measured as the turbidity change during the gel clotting in the reaction of the LAL reagent and endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® (ET-201, Wako Pure Chemical Industries).[42,43] The gelation time was defined by the reduction of the transmittance at 660 nm to 95% of the initial value according to the instruction manual.

Measurement of LPS in an HbV Suspension (Method 1) and Confirmation of its Validity

The HbV suspension ([lipid] = 6.0 g/dL, [Hb] = 10 g/dL) was put in LPS-free glass tubes, diluted with water for injection, mixed with a $C_{12}E_{10}$ solution, and then heated at 42°C for 2 min, as shown in Figure 1. The solution was then diluted eight times with water for injection. The LAL reagent (Wako Pure Chemicals Industries) was then added to the solution, and the LPS content was measured as the turbidity change in gel-clotting in the LAL–endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® ET-201.

To evaluate the validity of the Method 1, the recovery of spiked LPS was measured. An LPS standard solution ([LPS] = 0.4, .25, 2.5, 5.0, and 10.0 EU/mL) was added to the powdered lipid to adjust the lipid concentration to 6.0 g/dL. The suspension was agitated, diluted five times with the water for injection, and then freeze-thawed three times to prepare the LPS-contaminated vesicles. The recovery ratio was calculated by comparing the LPS concentration measured by the method already described and the spiked LPS concentration. A similar experiment was performed with HbV. An HbV suspension ([Hb] = 4 g/dL)

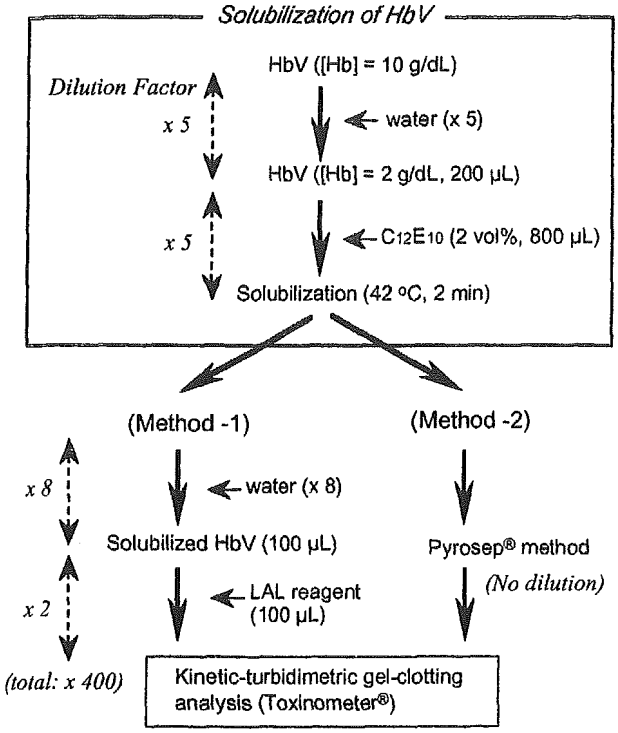

Figure 1. Procedures to quantitatively measure LPS in the HbV suspension using $C_{12}E_{10}$ for HbV solubilization and LAL assay, with dilution factors at every mixing of solutions. Method 1 is the condition for Entry 1 in Table 1. The Pyrosep® method (Method 2) does not have a dilution after solubilization of HbV.

was mixed with an equal volume of the LPS standard solution ([LPS] = 0.04, 0.16, 0.4, and 4.0 EU/mL) to prepare the LPS-spiked HbV ([Hb] = 2 g/dL), and solubilized by $C_{12}E_{10}$ for LAL assay as already described. As for the Hb solution, the same LPS standard solution ([LPS] = 0.002, 0.008, 0.02, 0.2, and 2.0 EU/mL) was mixed with an equal volume of an Hb solution ([Hb] = 4 g/dL) to prepare the LPS-spiked Hb solution ([Hb] = 2 g/dL), which was directly mixed with the LAL reagent to measure the LPS concentration. The recovery ratio was calculated by comparing it to the spiked LPS.

To confirm the effectiveness of the $C_{12}E_{10}$ treatment, LPS-contaminated vesicles were tested by Method 1 with or without $C_{12}E_{10}$. The vesicles were prepared by the addition of an LPS solution (1.0 EU/mL) to the powdered lipids (1.2 g/dL). The resulting suspension was freeze-thawed three times to enhance the dispersion of the lipids and to regulate the particle diameter to ~500 nm. The LPS concentration was measured, as already mentioned, using $C_{12}E_{10}$, and also without the addition of the $C_{12}E_{10}$ solution.

LPS Measurement in $C_{12}E_{10}$-Solubilized HbV using Histidine-Immobilized Agarose Gel (Pyrosep®, Method 2)

The *Limulus* PS Single Test Wako (Wako Pure Chemicals Industries) was used. A 5-mL aliquot of the $C_{12}E_{10}$-solubilized HbV solution ([Hb] = 0.4 g/dL, $[C_{12}E_{10}]$ = 1.6 vol%) was injected into a glass capillary column that contained 0.7 mL of histidine-immobilized agarose gel (Pyrosep®) to adsorb the LPS into the gel. The glass capillary column has a filter at the connected end of a silicone tube to retain the gel but allows permeation of the solution by aspirating air through the silicone tube. The gel was washed with 2 mL of LPS-free water to remove all the solutes except LPS, and then 0.3 mL of LAL-ES reagent (*Limulus polypherus* amebocyte lysate lyophilized, containing Tris-HCl buffer and β-1,3-glucan derivative) was injected into the capillary. All of the suspension was immediately pushed back into a glass vial, and the gelation time of the suspension was measured with a Toxinometer® (ET-201or ET-301 BL) with an extended MT-358 analysis module (Wako Pure Chemicals Industries). The time course of the transmittance change was detected at 660 nm, as already described. The inhibition/enhancement testing was performed to confirm the recovery ratio of the spiked LPS to the HbV in the same manner as already described.

RESULTS

Interaction of LPS with Phospholipid Vesicles Measured by Isothermal Titration Calorimetry (ITC)

ITC of the LPS injection showed exothermic peaks for every injection into the vesicles suspension, and the enthalpy change (ΔH) was calculated to be ~−80 kcal/mol (Fig. 2). As a reference experiment, the LPS solution was injected into pure water, and it was confirmed that the thermodynamic change was negligibly small (data not shown). The maximum amount of incorporation was 7.6 mol% into the outer surface of the vesicles under the assumption that the lamellarity of the vesicles was 2.

Solubilization of HbV with $C_{12}E_{10}$ Surfactant

The absorbance of the HbV suspension at 660 nm was 1.13, which is significantly higher than that of the Hb solution (0.08) due to the light scattering effect of the HbV particles. However,

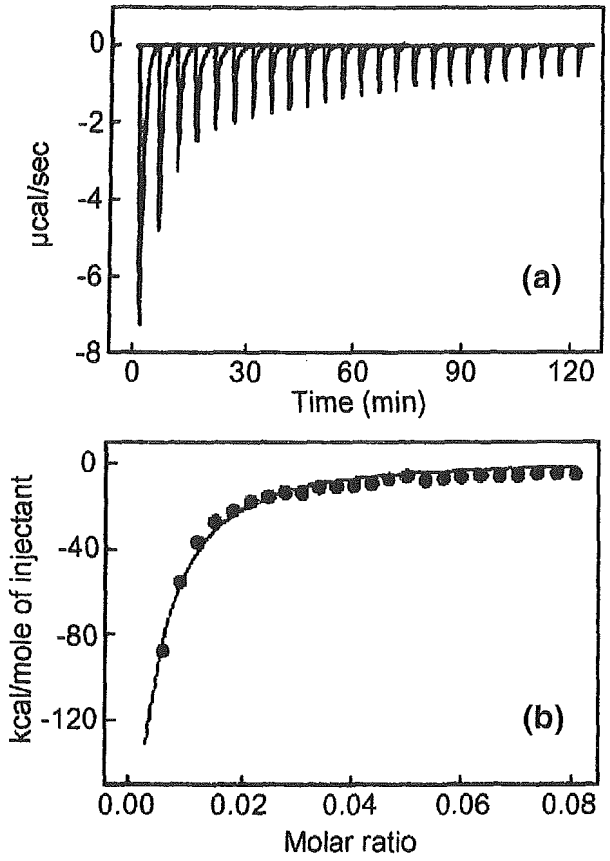

Figure 2. (a) Raw and (b) integrated data of the isothermal titration calorimetry for the incorporation of LPS (*Salmonella Minnesota* wild type) into phospholipid vesicles: [LPS] = 334 μM in a syringe (10 μL/one injection), [lipid] = 835 μM in a cell.

this absorbance decreased with the addition of the $C_{12}E_{10}$ solution, indicating the solubilization of HbV with the surfactant (Fig. 3). The complete solubilization required the addition of 2 vol% of $C_{12}E_{10}$ solution. At this concentration, the absorbance at 660 nm was identical with the pure Hb solution without vesicles. After the solubilization procedure, the color of the solution changed from red to brown, indicating the formation of methemoglobin. The concentration of $C_{12}E_{10}$ and the lipids of HbV in the resulting solution are 1.6 and 0.24 g/dL, respectively. $C_{12}E_{10}$ shares 87 wt% (86 mol%) of the mixed micelles. As for the other surfactants, $C_{12}E_9$ showed a similar ability to dissolve HbV that was evident from the profiles of the reduction of the light scattering of HbV (Fig. 3). On the other hand, Triton-X and sodium dodecyl sulfate (SDS) showed incomplete reduction of the absorbance and they could not sufficiently dissolve HbV.

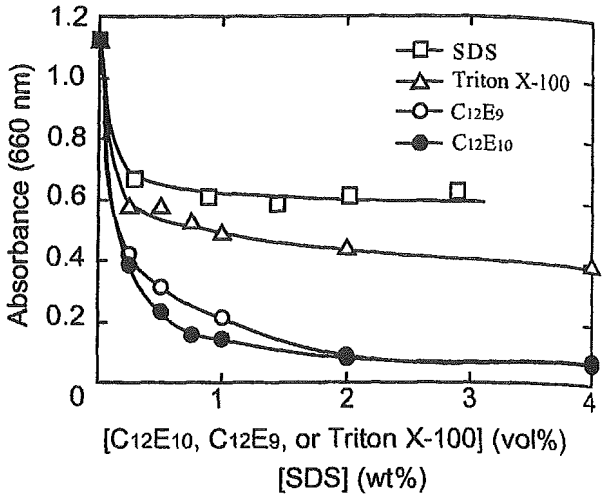

Figure 3. Absorption changes at 660 nm of HbV after solubilization of HbV by the addition of surfactants ($C_{12}E_{10}$, $C_{12}E_9$, Triton-X100, and SDS). Various concentration of a surfactant (800 μL) was added to the HbV suspension ([Hb] = 2 g/dL, 200 μL) and incubated at 42°C for 2 min.

Effect of $C_{12}E_{10}$ on Calibration Curves for LPS in LAL Assay (Method 1)

The calibration curves for Method 1 were drawn as logarithm plots of the gelation time versus the final LPS concentration (Fig. 4). Actually, the

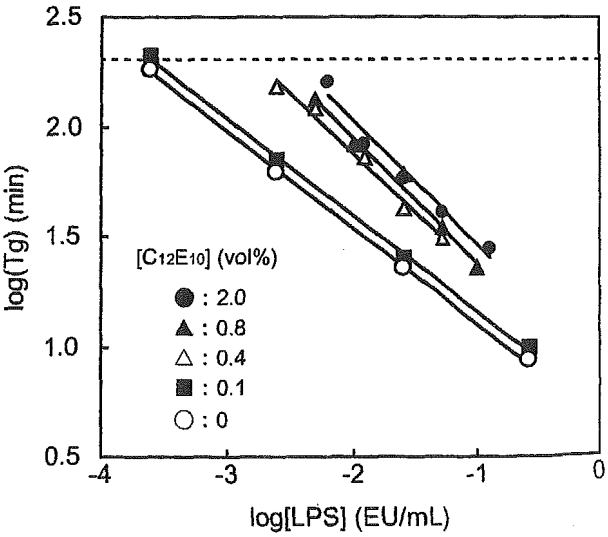

Figure 4. Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations. [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dL. For example, [LPS] should be multiplied with 400 at $[C_{12}E_{10}] = 0.1$ wt%. The broken line indicates the gelation time limit for Toxinometer® (200 min).

plots show a linear relationship over a wide range of LPS concentrations (final concentration: 0.000125–0.25 EU/mL). The gelation time limit for the Toxinometer® is 200 min (log 200 = 2.3) as shown by the dashed line. Increasing the $C_{12}E_{10}$ concentration tended to retard the gelation. However, 0.1 vol% of the final $C_{12}E_{10}$ concentration did not show a significant retardation. At the LPS concentration of 0.01 EU/mL, the gelation time of ~32 min without $C_{12}E_{10}$ was prolonged to 39 min with 0.1 vol% of $C_{12}E_{10}$ and to ~107 min with 2.0 vol% of $C_{12}E_{10}$. In the case of the other surfactant, the presence of 0.1 vol% $C_{12}E_9$ prolonged the gelation time to 50 min (data not shown). Therefore, the inhibitory effect of $C_{12}E_9$ is stronger than that of $C_{12}E_{10}$.

LPS Measurement in HbV (Method 1)

To improve the LPS detection limit, the dilution factor could be minimized by increasing the HbV concentration at the solubilization process or by decreasing the amount of diluent (Table 1). The lowest detection limit (0.1 EU/mL) was obtained for entry No.1, with 0.1 vol% $C_{12}E_{10}$. The conditions of entries 2, 3, and 4 could reduce the dilution factor, however, [$C_{12}E_{10}$] at >0.4 vol% resulted in a significant inhibition of the gelation, as shown in Figure 4 and by the worsened detection limit (0.23 EU/mL).

The recoveries of the spiked LPS ranged between 92 and 124% for a wide range of LPS concentrations (Table 2, next page). The LPS measurement in an Hb solution did not require a surfactant so it was possible to detect a lower concentration (0.005 EU/mL), which was significantly better than the detection limit for HbV (0.1 EU/mL) that required $C_{12}E_{10}$. The FDA Guideline, published in 1987, requires that the inhibition/enhancement testing of spiked LPS at four times the amount of the detection limit and that the recovery should be within 100 ± 25%.[17] We tested the addition of LPS at 0.4 EU/mL (= 4 × 0.1 EU/mL) to the phospholipid vesicles and HbVs and at 0.02 EU/mL (= 4 × 0.005 EU/mL) to the Hb solution, and all the recoveries were within 100 ± 25%.

The LPS content in the LPS-contaminated vesicles, measured with the aforementioned LAL assay using $C_{12}E_{10}$, was 1.03 EU/mL (Table 3). On the other hand, the LAL assay without $C_{12}E_{10}$ resulted in 0.6 EU/mL. The recovery was calculated to be 60%.

The Pyrosep® Method to Detect LPS in $C_{12}E_{10}$-Solubilized HbV (Method 2)

The calibration curves of the Pyrosep® method in the presence of $C_{12}E_{10}$ showed a detection limit of 0.0001 EU/mL or lower (Fig. 5). The dilution factor for the HbV measurement was 25. Therefore, the LPS detection limit for an HbV suspension at [Hb] = 10 g/dL is 0.0025 EU/mL. One HbV suspension showing an LPS content of <0.1 EU/mL as measured by Method 1 was shown to have an LPS content of 0.011 EU/mL by the Pyrosep® method; this HbV was used for the inhibition/enhancement testing. The recovery of spiked LPS (Fig. 5, inset; 0.001, 0.01, and 0.1 EU/mL) from an HbV suspension was within 100 ± 25% over a wide range of LPS concentrations.

DISCUSSION

The interaction between an amphiphilic LPS molecule and a phospholipid vesicle should influence the accuracy of the LAL assay. It has been

Table 1. Solubilization Condition of HbV with $C_{12}E_{10}$ and Detection Limit of LPS[a]

Entry	Condition of HbV Solubilization: HbV/200 μL ([Hb] g/dL)	Condition of HbV Solubilization: $C_{12}E_{10}$/800 μL ([$C_{12}E_{10}$] vol%)	Dilution Factor[b]	Final vol% [$C_{12}E_{10}$]	Detection Limit of LPS (EU/mL HbV)[c]
1[d]	2	2	400	0.1	>0.1
2	2	2	100	0.4	>0.25
3	2	2	50	0.8	>0.25
4	5	5	20	2.0	>0.23

[a]The calibration curves are shown in Figure 4.
[b]See Figure 1, from HbV ([Hb] = 10 g/dL) to gel clotting assay.
[c]At [Hb] = 10 g/dL.
[d]The optimal condition in this study.

Table 2. Inhibition/Enhancement Testing to Monitor the Recovery of Spiked LPS

Sample	Spiked LPS (EU/mL)[a]	Recovery (%)
Vesicles (6 g/dL) with $C_{12}E_{10}$	0.4	108.6 ± 4.6[b]
	1.25	92
	2.5	116
	5	103
	10	120
HbV (Hb, 10 g/dL; lipid, 6 g/dL) with $C_{12}E_{10}$	0.1	107
	0.4	111.3 ± 3.6[b]
	1.0	114
	10.0	112
Hb solution (10 g/dL) without $C_{12}E_{10}$	0.005	121
	0.02	103.9 ± 4.4[b]
	0.05	101
	0.5	124
	5	97

[a]LPS was spiked in the $C_{12}E_{10}$-solubilized vesicles and HbV, and Hb solution without $C_{12}E_{10}$. All the spiked LPS concentrations are converted to the conditions of [Hb] = 10 g/dL, or [lipid] = 6 g/dL. All the recoveries were within $100 \pm 25\%$.
[b]$n = 3$.

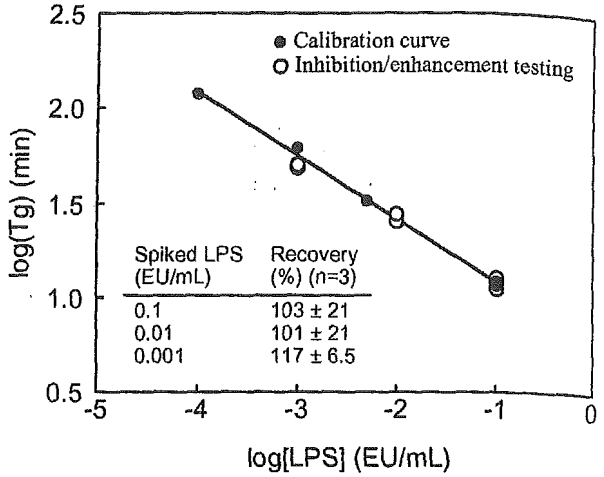

Figure 5. A calibration curve for the Pyrosep® method including the treatment of $C_{12}E_{10}$, and the results of the LPS-spiking test to the HbV suspension. Inset: The recovery of the spiked LPS in the HbV suspension. During the procedure, the HbV suspension was diluted 25 times. Therefore, the spiked LPS concentrations of 0.1, 0.01, and 0.001 EU/mL correspond to 2.5, 0.25, and 0.025 EU/mL, respectively, at [Hb] = 10 g/dL. The original LPS content in the HbV suspension was 0.011 EU/mL at [Hb] = 10 g/dL.

well documented that the biological activity of the LPS is significantly suppressed when an LPS molecule is incorporated into phospholipid vesicles.[25,26,44,45] However, there has been no report on the quantitative observation of the LPS–phospholipid vesicle interaction. Using the ITC method, we quantitatively clarified for the first time that LPS from the *Salmonella Minnesota* wild type (smooth form) was inserted into the phospholipid vesicles with an enthalpy change (ΔH) of ~ -80 kcal/mol and the maximum incorporation of ~ 7.6 mol% on the outer surface of the vesicles. To our knowledge, the ΔH value of PEG_{5000}–DSPE (MW of PEG = 5 kDa) for the same phospholipid vesicles is only -13 kcal/mol.[38]

Table 3. Detection of LPS in the LPS-Contaminated Vesicles with or Without the $C_{12}E_{10}$ Treatment[a]

Treatment	LPS (EU/mL)	Recovery (%)
Without $C_{12}E_{10}$	0.6	60
With $C_{12}E_{10}$	1.03	103

[a][lipid] = 1.2 g/dL, [spiked LPS] = 1.0 EU/mL. The higher recovery of the $C_{12}E_{10}$-solubilized vesicles indicates the necessity of the pretreatment.

This comparison indicates that LPS inserted into the bilayer membrane is thermodynamically more stabilized than PEG_{5000}–DSPE. The large difference in ΔH is probably due not only to the hydrophobic interactions by the eight alkyl chains of LPS but also to the hydrogen bonding of the amide bonds into the interface of the hydrophobic and hydrophilic regions that contribute to the interaction of LPS with the phospholipid bilayer membrane. As for the interaction between Hb and LPS, the ITC analysis was recently reported by Jurgens et al.,[46] who clarified that 3–5 LPS molecules bind to one Hb molecule.

These ITC results clearly emphasize the necessity of the pretreatment of HbV for the LAL quantitative measurement of LPS; that is, to release LPS from the phospholipid vesicles using an organic solvent[47] or surfactant.[28,29] We tested the solubilization of HbV with a surfactant because of the presence of a large amount of Hb that is insoluble in an organic solvent. We determined the optimal condition as Method 1 to detect the LPS content, with the best detection limit at 0.1 EU/mL for HbV at [Hb] = 10 g/dL. The lipid bilayer of HbV is composed of DPPC/cholesterol/DPEA/PEG_{5000}–DSPE at a molar ratio of 5:5:1:0.033, and the vesicles are quite stable due to the

high phase transition temperature (T_c) of DPPC (41°C), the improved molecular packing using cholesterol, the repulsive force between the bilayer membrane due to the presence of a negatively charged DPEA, and the steric hindrance of the PEG chains of PEG–DSPE. However, the procedure of the $C_{12}E_{10}$ addition and incubation at 42°C (near T_c) for 2 min completely diminished the light scattering of HbV, and this condition can effectively solubilize the HbV.

We confirmed that $C_{12}E_{10}$ showed a significant inhibitory effect at a higher concentration that is probably due to the inhibition of gel formation or solubilization of the gelated coagulin (Fig. 3). However, the final concentration of $C_{12}E_{10}$ at 0.1 vol% showed a moderate inhibitory effect. As for the other surfactants, nona(oxyethylene) dodecyl ether ($C_{12}E_9$) showed a more significant inhibitory effect for the LAL assay even though it showed a sufficient solubilization of HbV, and Triton-X 100 showed a lower solubilization of HbV. An anionic surfactant, SDS, did not show complete HbV solubilization. Moreover, according to the literature, SDS completely inhibits gelation only at >0.005 wt%.[29] On the contrary, nonionic surfactants, especially $C_{12}E_{10}$, moderately decrease the LAL activity in relation to the surfactant concentration. $C_{12}E_{10}$ has a sufficient ability to dissolve vesicles and a moderate inhibitory effect on the LAL assay. The solubilization of HbV with $C_{12}E_{10}$ should lead to the formation of micellar structures containing $C_{12}E_{10}$, LPS, and the lipid components. $C_{12}E_{10}$ shares 86 mol% of the components of the micelles, which is significantly higher than PEG–DSPE (0.04 mol%), and the aggregation number of the $C_{12}E_{10}$ micelle is estimated to be ~100.[41] Therefore, the LAL proteins could interact with the lipid-A moiety at the interface of the aqueous and hydrophobic phases of the micelles without the excluded volume effect of the PEG–DSPE.

Improvement of the detection limit requires a lower dilution factor and lower $C_{12}E_{10}$ concentration. However, a reduction in the dilution factor is accompanied by an increase in the concentration of $C_{12}E_{10}$. When the [$C_{12}E_{10}$] was 0.1 vol%, the inhibitory effect was minimal (see Fig. 4), and we obtained the optimal pretreatment condition as entry No. 1 in Table 1. The total dilution factor of this condition is 400. The FDA Guideline defines the maximum valid dilution (MVD) depending on the maximum dose (mL/kg) and lysate sensitivity.[17] The MVD for HbV should be 1000 (= 0.25/0.00025) under the condition that the LPS limit for HbV is 0.25 EU/mL, and the detection limit in the standard curve is 0.00025 EU/mL [log(0.00025) EU/mL = −3.60; (see Fig. 4). Therefore, the dilution factor of 400 is within the MVD.

We compared the measurement of the LPS content in the vesicles with or without $C_{12}E_{10}$ and found that the solubilization of vesicles with $C_{12}E_{10}$ showed a higher LPS concentration. This result clearly demonstrates that LPS molecules in the bilayer membrane or in the inner aqueous phase of the vesicles are released and detected after the solubilization. The recovery of spiked LPS was reproducible within the range 92–120% over a wide range of LPS concentrations. The FDA Guideline requires LPS recovery in the range of 100 ± 25% to indicate no influence of solutes in a specimen.[17] This value was revised to 100 ± 50% in 1991.[48] In spite of the ITC analysis of Hb that indicated 3–5 LPS molecules bind to one Hb,[46] we did not see any interference effect by the presence of Hb. Our result is in contrast to previous reports. Levin et al.[22,49] reported that LPS bound to Hb showed activation of LAL, whereas Archambault et al.[50] reported a significant reduction of LAL activity when the LPS was bound to Hb. Jurgens et al.[46] reported that LAL activity depended on the concentrations of LPS and Hb in the assay. The reason for these discrepancies is not clear, however, there is a large difference in the LPS concentrations for the LAL assay between the reports. In our assay conditions, we measured the LPS concentration at <0.2 EU/mL (~20 pg/mL). On the other hand, other groups conducted the experiments at much higher LPS concentrations (100 pg/mL–800 μg/mL). In some cases, the LPS concentrations were above the critical aggregation concentrations (10–38 μg/mL).[40] It was reported that the LAL activity was significantly suppressed by the LPS aggregation.[51] In the presence of Hb, LPS should bind to Hb rather than form aggregates, resulting in an apparent enhancement of the LAL activity. On the contrary, in our significantly diluted assay condition, it can be speculated that LPS should not be aggregated and be more freely dispersed from Hb, and the presence of Hb should not induce any inhibition or enhancement.

The LPS measurement is often performed to monitor patients with septic shock. Because blood plasma contains some unknown elements that inhibit or accelerate the LAL assay, Obayashi et al.[52] proposed perchroic acid (PCA) treatment to inactivate or remove the interfering plasma components and to obtain a sufficient recovery of

the spiked LPS. In this method, the proteins denatured by PCA are removed as a precipitate. However, this method leads to underestimation of the LPS content because LPS strongly binds to plasma proteins, such as albumin, lipoprotein, and the LPS-binding protein,[53] which should denature and precipitate after the PCA. The new PCA method[54] includes the addition of an alkaline solution to solubilize the denatured protein so that the recovery of LPS is much improved. The dilution of plasma with water and subsequent heating at 100°C for 10 min in the presence of a weak surfactant such as Triton-X100 also releases LPS from the plasma protein.[42,45] On the other hand, our HbV does not contain plasma-derived interfering elements because it is made from ultrapurified Hb solution. During the solubilization of HbV with $C_{12}E_{10}$ at 42°C for 2 min, Hb is partly oxidized to form metHb. However, there is no further denaturation of metHb and its precipitate. Moreover, a derivative of β-1,3-glucan, which is well-known as a significant interfering element for the LAL assay,[55,56] is intentionally added to the LAL reagent from Wako Pure Chemicals Industries, so the influence of contaminated β-1,3-glucan in a specimen is eliminated.[57] For all the pretreatments, the kinetic–turbidimetric assay system can be used for the LAL clotting assay using the Toxinometer®.[43,58] In the case of Hb and HbV, the strong absorption band of the Hb molecule between 400 and 600 nm may affect not only the turbidimetric measurement but also the chromogenic measurement with detection wavelengths of 405 or 545 nm (e.g., Endospecy, Seikagaku Kogyo, Ltd., Japan). In this sense, the detection wavelength of 660 nm using the Toxinometer® should be appropriate.[58]

The Pyrosep® method (Method 2) was very effective in detecting trace amounts of LPS in the $C_{12}E_{10}$-solubilized HbV samples. The solubilization of HbV requires a dilution factor of 25 as in Method 1 (see Fig. 1). There is no additional dilution afterward because the solubilized HbV was treated with the Pyrosep® column and the LPS-bound agarose gel was directly mixed with the LAL reagent. As a result, the lowest LPS concentration for the calibration curve was 0.0001 EU/mL, with a dilution factor of only 25; therefore, the detection limit was calculated to be 0.0025 EU/mL in HbV at [Hb] = 10 g/dL. The LPS content of one HbV suspension was determined to be <0.1 EU/mL, by Method 1, whereas it was 0.011 EU/mL as measured by the Pyrosep® method. This latter method also showed a sufficient recovery of the spiked LPS for a wide range of LPS concentrations. Not only the electrostatic interaction between the cationic region of the histidine residue and the anionic region of LPS, but also the hydrophobic interaction between the spacer region of the histidine–agarose conjugate and alkyl chains of LPS should contribute to the specific adsorption of LPS on the agarose gel.[30] After the adsorption of LPS, the agarose gel was washed with LPS-free water so that all the interfering elements, such as a surfactant, were removed before reacting with LAL reagent. Because Hb was also washed out, the detection wavelength was not limited to 660 nm to avoid the absorption band of Hb. As far as we know, this is the first attempt to measure the LPS content by the combination of Pyrosep® and surfactant pretreatment.

This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer® is routinely used in our production system of HbVs. Significant attention is paid to the quality control of HbVs for preclinical studies, and all the HbVs prepared under sterile conditions showed an LPS content of <0.1 EU/mL at [Hb] = 10 g/dL. Moreover, utilization of the Pyrosep® improved the detection limit to 0.0025 EU/mL. Our method enables an accurate measurement of trace amounts of LPS in HbVs as an oxygen carrier of which the dose rate should be significantly large in comparison with the conventional drugs. Of course, this method is applicable to the quality control of other phospholipid vesicles and also protein drugs such as albumin and their recombinant types that strongly bind LPS.

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