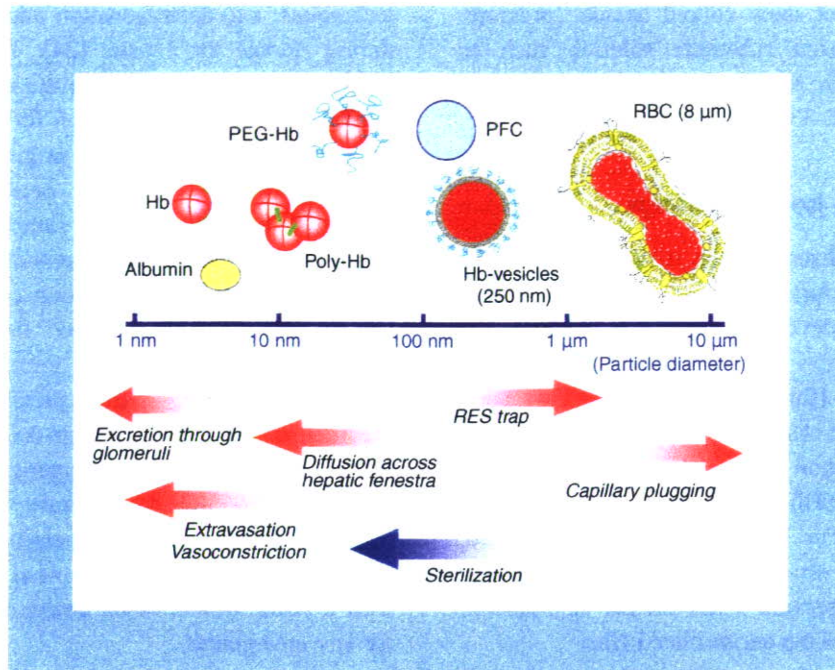

Fig. 1 What is the optimal dimension of artificial oxygen carriers? There is an upper limitation, below the capillary diameter, to prevent capillary plugging, and for the sterilization by membrane filters. On the other hand, the much smaller ones show higher rates of renal excretion and vascular wall permeabilities with side effects such as hypertension and neurological disturbances. Hb-vesicles show very low level of vascular wall permeabilities. Therefore, the Hb-vesicles seems appropriate from the viewpoint of hemodynamics. However, we have to clarify the influence of Hb-vesicles on the reticuloendothelial system (RES) because the fate of Hb-vesicles is RES trapping (see Fig. 3).

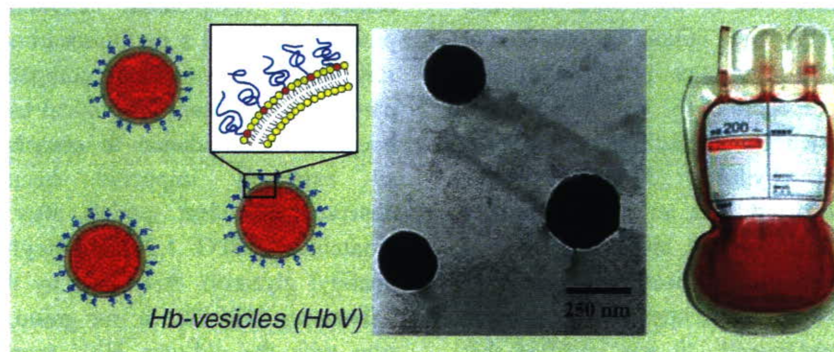

Fig. 2 (Left) Schematic representation of Hb-vesicle (HbV). One particle contains about 30 000 Hb molecules. The surface of one HbV is modified using polyethylene glycol chains that ensure the dispersion stability of HbV during storage and during circulation in the bloodstream. (Middle) The transmission electron micrograph depicts the well-regulated particle size (250 nm) and high Hb content within the vesicles. (Right) The packed HbV suspension looks turbid, like a mixture of milk and red wine, because of light-scattering of the particle suspension.

5 The cellular structure, which resembles that of RBCs, shields all side effects of Hb molecules, such as scavenging NO and CO [8, 9, 27];

6 The particle size (250 nm) is appropriate for sterilization, circulation persistence and biodistribution [18, 28] and

7 Hb-vesicles do not show colloid osmotic pressure. Addition of a plasma substitute solution such as recombinant albumin is effective to regulate colloid osmotic pressure [31–33].

Stabilized HbV for a long-term storage

Because Hb autoxidizes to form metHb and loses its O_2-binding ability during storage as well as during blood circulation, prevention of metHb formation is necessary. Some groups have reported a method to preserve deoxygenated Hbs in the liquid state [34] using well-known intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the O_2 partial pressure; also, deoxyHb is not autoxidized at ambient temperatures [35]. For HbV, not only the inside Hb, but also the cellular structure (liposome) must be physically stabilized to prevent intervesicular aggregation, fusion and leakage of the encapsulated Hb.

Liposomes, as molecular assemblies, have been generally inferred to be structurally unstable. Many researchers have sought to develop stabilization methods that use polymer chains [36]. Polymerization of phospholipids that contain dienoyl groups was studied extensively in our group. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in phospholipids. This method produces enormously stable liposomes, like rubber balls, which are resistant to freeze-thawing, freeze-drying and rehydration [37–39]. However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages, even 30 days after injection [40]. It was concluded that polymerized lipids would not be appropriate for intravenous injection. Subsequently, it was clarified that selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition are important to enhance the stability of liposomes without polymerization. Surface modification of liposomes with PEG chains is sufficient for dispersion stability [24–30].

We investigated the possibility of long-term preservation of HbV through a combination of two techniques, e.g. deoxygenation and PEG modification during storage for 2 years [24]. The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion for 2 years because of their steric hindrance. The original metHb content (approximately 3%) before preservation decreased gradually to <1% in all samples after 1 month because of the presence of a reductant, such as homocysteine, inside the vesicles that consumed the residual O_2 and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the O_2 partial pressure: no increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for 2 years. These results clearly indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency.

Blood compatibility of Hb-vesicles

Liposome is not a solute but a particle in a suspension. Once injected, the surface is sometimes recognized by, or interacted with blood components. The so-called 'injection reaction', or pseudo-allergy is caused by complement activation with liposomal products [41] and a perfluorocarbon emulsion. Therefore, examination of blood compatibility of liposomal particles is important for clinical use. Transient thrombocytopenia in relation to complement activation is an extremely important haematological effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol), developed by the Naval Research Laboratory [42, 43]. In our group, exchange transfusion with the old-type HbV (containing DPPG, no PEG modification) in anesthetized rats resulted in thrombocytopenia [31]. Similar effects were also observed for administration of negatively charged liposomes [44, 45]. The transient reduction in PLT counts caused by liposomes was also associated with sequestration of PLTs in the lung and liver. Such nonphysiological PLT activation would engender initiation and modulation of inflammatory responses because PLTs contain an array of potent proinflammatory

substance. However, the present HbV apparently does not induce thrombocytopenia in animal experiments, probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate), not DPPG or a fatty acid [22, 23].

Detailed blood compatibility of HbV in relation to negatively charged lipid was examined by Dr H. Ikeda at Hokkaido Red Cross Blood Center (Sapporo) and his colleagues [22, 23, 25, 46]. The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, whereas HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was activated slightly by DPPG-HbV, but not by the present PEG-DHSG-HbV. Moreover, the complement consumption of the plasma was observed by incubation with DPPG-HbV, but not with the present PEG-DHSG-HbV. These results indicate that the present PEG-DHSG-HbV has a higher biocompatibility with human plasma. Moreover, the exposure of human PLTs to high concentrations of the present HbV (up to 40%) *in vitro* did not cause PLT activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by PLT agonists. These results imply that HbV, at concentrations of up to 40%, has no aberrant interactions with either unstimulated or agonist-induced PLTs.

Biodistribution and fate of Hb-vesicles in reticuloendothelial system

The dose rate of blood substitutes would be considerably larger than those of other drugs, and their circulation time would be considerably shorter than RBC. Therefore, their biodistribution, metabolism, excretion and side effects must be characterized in detail especially about the reticuloendothelial system (RES).

Normally, free Hb released from RBC is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin-binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Haemoglobinuria and eventual renal failure occur when the reabsorption capacity of the kidney is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES (or mononuclear phagocytic system) in much the same manner as senescent RBC are, as confirmed by radioisotope ^{99m}Tc-labelled HbV injection [15, 28]. Gamma camera images of ^{99m}Tc-HbV showed that HbV remains in the bloodstream immediately after infusion so that the heart and liver that contain much blood showed strong intensity (Fig. 3a). However, HbV are finally distributed mainly in the liver, spleen and bone marrow. The circulation half-life is dose dependent; when the dose rate was 14 mL kg^{-1}, the circulation half-life was 32 h. The circulation time in the case of the human body can be estimated as twice or three times longer; or about 2 or 3 days at the same dose rate.

The time course of liver uptake was monitored using a confocal fluorescence microscope after fluorescence-labelled HbV was infused intravenously in an anesthetized hamster. Even though the individual particles of HbV were indistinguishable, they are recognizable with strong fluorescence when HbV are accumulated in phagosomes of Kupffer cells (Fig. 3b). Transmission electron microscopy (TEM) of the spleen 1 day after infusion of HbV clearly demonstrated the presence of HbV particles in macrophages, where HbV particles that appear as black dots are captured by the phagosomes [47] (Fig. 3c). However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. Immunochemical staining with a polyclonal anti-human Hb antibody was used as the marker of Hb in the HbV, and clarified that HbV almost disappeared after 7 days in both the spleen and liver (Fig. 3d) [47].

During metabolism of Hb, bilirubin and iron would be released. However, in our animal experiments of topload infusion, daily repeated infusions, and 40% blood exchange, neither of those products increased

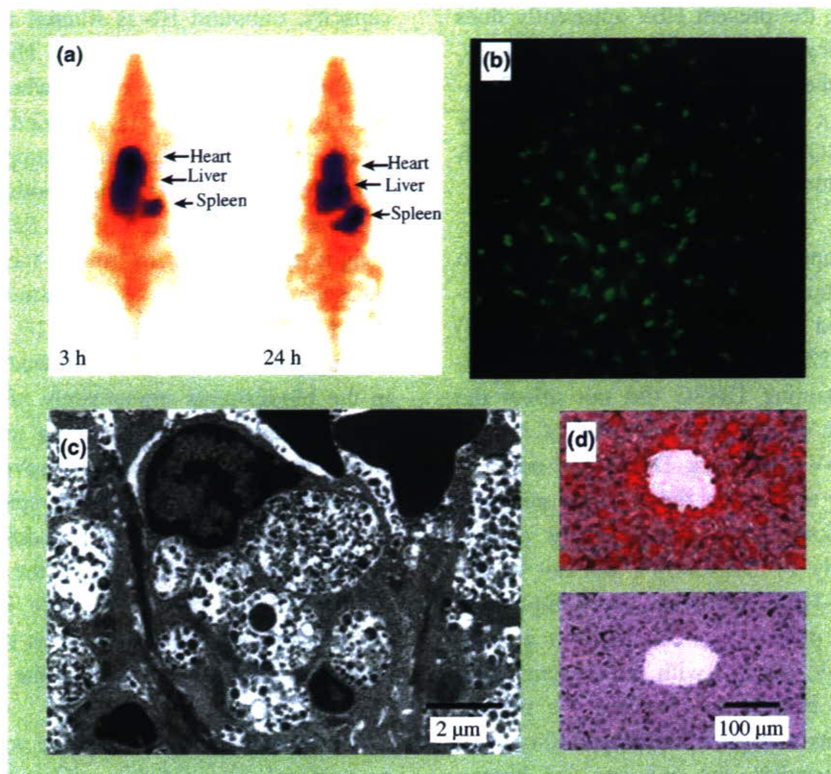

Fig. 3 Biodistribution and fate of Hb-vesicle (HbV). (a) Gamma-camera images of the distribution of ^{99m}Tc-labelled HbV in rats. At 3 h after injection, the heart and the liver showed a strong intensity because of the large blood volume. However, 24 h later, the intensity increased in the liver and spleen, the so-called reticuloendothelial system. (b) The liver surface of an anesthetized golden hamster 40 min after injection of fluorescence-labelled HbV observed using laser confocal scanning microscopy. The individual HbV particles flowing in the sinusoid are not detected, but the strong fluorescence is observed only in the Kupffer cells when they phagocyte HbV. (c) Transmission electron micrograph of rat spleen 1 day after intravenous injection of HbV. The small black dots are HbV near red blood cell in the capillaries and in the phagosomes of spleen macrophages. They disappear completely within 1 week. (d) Staining with anti-human Hb antibody revealed the presence of HbV in the liver Kupffer cells and sinusoids 1 day after infusion. However, they disappear within 1 week.

in the plasma within 14 days [33, 48, 49]. The released haeme from Hb in HbV might be metabolized by the inducible form of haeme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages. Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Berlin blue staining revealed considerable deposition of haemosiderin in the liver and spleen, even after 14 days. Normally, iron from a haeme is stored in the ferritin molecule. Both ferritin and haemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation. The iron release rate from haemosiderin, however, is substantially less than that from ferritin. Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as haemosiderin. Hemosiderosis often occurs in patients who have received repeated blood transfusions because of the shorter half-life of the stored RBCs. Moderate splenomegaly and haemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with the lowered membrane deformability and shortened circulation half-life [33, 50].

As for the membrane components of HbVs, it was reported that the infused lipid components of

liposomes are entrapped in the Kupffer cells, and that phospholipid is metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and CO_2 in exhaled air. It was recently clarified using a ^{3}H-cholesterol that cholesterol of HbV is released from macrophages to blood, and is ultimately excreted in faeces. The PEG chain is widely used for surface modification of liposomal products. The chemical crosslinker of PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which are known as inert macromolecules, should be excreted in urine through the kidneys [51].

More precise data are necessary. However, these results imply that the metabolism of HbV and the excretion are within the physiological capacity that has been well characterized for the metabolism of senescent RBCs and conventional liposomal products.

Rheological properties and efficacy of an Hb-vesicle suspension as a transfusion alternative

A single HbV particle (approximately 250 nm diameter) contains about 30 000 Hb molecules. The HbV is much smaller than RBC, PLT or white blood cell (WBC) particles (Fig. 4a). Nevertheless, HbV acts as a particle in the blood and not as a solute; the colloid osmotic pressure of the HbV suspension is nearly zero. Addition of a plasma expander is necessary for a large substitution of blood to maintain the blood volume. The plasma expander candidates are human serum albumin (HSA), hydroxyethyl starch, dextran or gelatine, depending on the clinical setting, cost, country and clinician. Recombinant human serum albumin (rHSA) is an alternative [32, 33]. The impossibility of transmission of any infectious disease from humans is the greatest advantage of rHSA, which will soon be approved for clinical use in Japan [52].

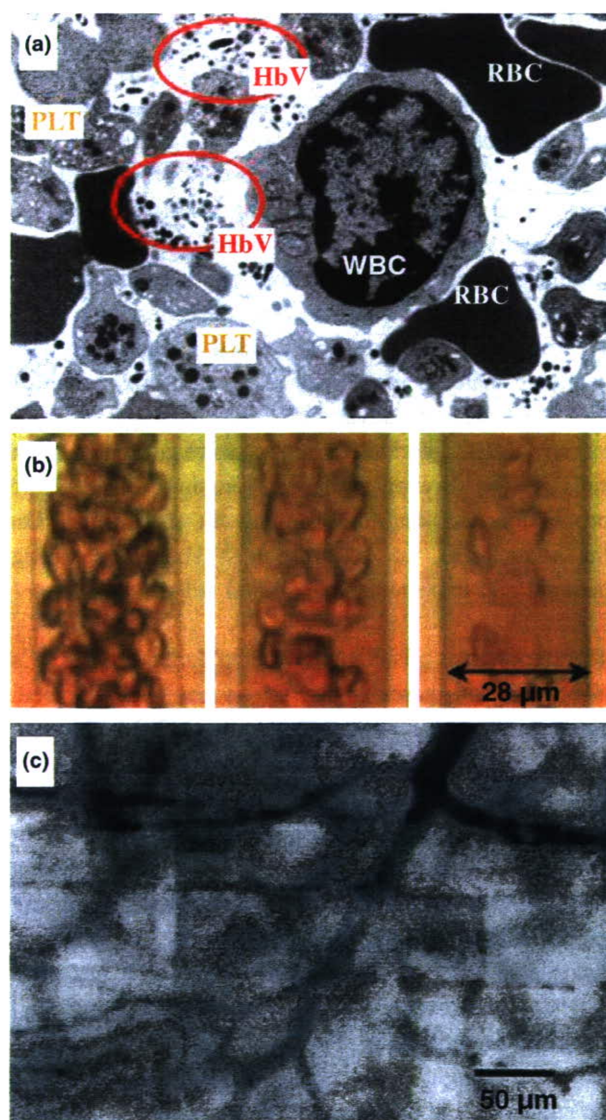

Fig. 4 How small is Hb-vesicle (HbV)? (a) The transmittance electron micrograph of rat blood 1 day after infusion of HbV. The buffy coat, obtained by a centrifugation of blood, was fixed using a 2.5% glutaraldehyde solution. Many HbV particles are visible in the red circles. They are much smaller than red blood cell (RBC), WBCs or PLT. (b) Flow patterns of the mixture of HbV and RBC suspended in recombinant human serum albumin in a narrow tube (centerline flow velocity: 1 mm s^{-1}). From left to right, the mixing ratios, RBC/HbV by volume are 100/0, 50/50 and 10/90 at a constant (Hb) = 10 g dL^{-1}. The RBCs tend to flow in the centerline, whereas HbV particles are dispersed homogeneously in a suspension medium. (c) Micrograph of a hamster skin microvasculature after 80% exchange transfusion with HbV suspended in 5% HSA solution, with an illumination with a wavelength of about 420 nm, being absorbed at the *Soret* band of Hb in HbV and RBC. The capillaries are blackened because of the homogeneous dispersion of HbV in the plasma phase. This homogeneous distribution believed to be effective for tissue oxygenation.

The rheological property of an artificial oxygen carrier is important because the infusion amount should be considerably large, which might affect the blood viscosity and hemodynamics. The viscosity of HbV suspended in 5%-rHSA was similar with that of blood, and the mixtures with RBC at various mixing ratios showed viscosities of 3–4 cP [53]. The main component to determine blood viscosity is RBC; the results indicate no great interaction between HbV and RBC. To observe the flow pattern of the mixture of HbV and RBC, they were mixed in various volume ratios. Then the suspension was perfused through an O_2-permeable narrow tube (28 μm inner diameter) and exposed to a deoxygenated environment [6]. Because HbV was dispersed homogeneously in the rHSA solution, increasing the volume of the HbV suspension thickened the marginal RBC-free layer and the plasma phase became semitransparent (Fig. 4b). The measurement of the O_2-release rate showed that HbV releases O_2 similarly to RBCs. On the other hand, an acellular Hb solution, in a comparative study, showed the facilitated O_2-release attributable to the effect of diffusion of small HbO_2. The slow O_2-release rate of HbV, which resembles that of RBC, is important to prevent autoregulatory vasoconstriction. Microvascular observation after 80% exchange transfusion with HbV suspended in HSA in conscious hamsters with a dorsal skin-fold window model of Prof. Intaglietta (UCSD) also showed that HbV was distributed homogeneously in the plasma phase; the capillary shape was visualized (Fig. 4c). This homogeneous distribution is inferred to be effective for improved blood flow and homogeneous tissue oxygenation.

Extensive *in vivo* studies of such HbV suspended in plasma-derived HSA or rHSA revealed sufficient O_2 transporting efficiency that is apparently comparable to RBCs in extreme blood exchange experiments [29–31, 33, 54–56] and fluid resuscitation from hemorrhagic shock [32, 57–60]. It was confirmed in rat models that haematopoietic activity was preserved and the decreased haematocrit returned to the original level within 1 or 2 weeks, whilst HbV captured in RES disappeared completely [33]. A recent experiment of HbV suspended in rHSA as a priming solution for cardiopulmonary bypass (CPB) in a rat model showed that HbV protects neurocognitive function by transporting O_2 to brain tissue even when the haematocrit is reduced markedly [61]. Homologous blood use is considered to be the gold standard for CPB priming in infants despite exposure of patients to potential cellular and humoral antigens. However, the results indicate that the use of HbV for CPB priming might prevent neurocognitive decline in infants because of considerable hemodilution. Other studies investigating HbV suspension as a possible perfusate for organ transplantation are also underway for the heart, liver, intestine, etc.

New concepts to design HbV

Development of artificial O_2 carriers was initiated originally with a simple idea and an expectation that the materials that bind or dissolve O_2 can behave similarly to RBCs in the bloodstream. Unfortunately, it was not so simple. During its long history of development, unexpected side effects were clarified such as capillary plugging, renal toxicity, vasoconstriction, vascular injury and accumulation. Decades-long R&D of artificial O_2 carriers has yielded no commercially available material for clinical use in Europe, Japan or the US. Recent advanced biotechnology enables *ex vivo* RBC production from haematopoietic stem cells [62]. However, problems remain of large-scale production and long-term storage for stockpiling. On the other hand, no doubts persist about the strong demand and expectation of a blood substitute.

The importance of the sophisticated function of RBCs in concert with vascular physiology has been clarified. New concepts are proposed in terms of the physicochemical properties of Hb-based artificial O_2 carriers. Historically, it has been regarded that the O_2 affinity is regulated similarly to RBCs (25–30 torr). Theoretically, this enables sufficient O_2 unloading during blood microcirculation, as can be evaluated according to the arterio-venous difference in O_2 saturation in accordance with an O_2 equilibrium curve. It has been expected that decreasing O_2 affinity (increasing P_{50}) increases O_2 unloading. However, this concept is controversial in light of recent findings because an excess O_2 supply would cause autoregulatory vasoconstriction

and microcirculatory disorders. A new conceptualization is that HBOCs with a high O_2 affinity (low P_{50}) retain O_2 in the upstream artery or arteriole and release O_2 in the capillaries of the targeted tissue. This hypothesis has been supported recently by results of PEG-modified Hbs and HbV by microcirculatory observations [55, 56, 63, 64]. The P_{50} of HbV is easily regulated by manipulating the content of an allosteric effector, pyridoxal 5′-phosphate (PLP), inside the HbV [55, 65]. For example, equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and P_{50} was regulated to 18 torr. When the molar ratio PLP/Hb was 3/1, P_{50} was regulated to 32 torr. The present HbV contains PLP at PLP/Hb = 2.5 by mol; the resulting P_{50} is about 25–28 torr, which shows sufficient O_2 transporting capacity as a transfusion alternative for extreme hemodilution, resuscitative fluid for hemorrhagic shock and prime solution for extracorporeal circulation. The P_{50} of HbV without PLP and Cl^- is 8–9 torr.

Because infusion of an artificial O_2 carrier necessitates the substitution of a large volume of blood, its impact on hemorheology is remarkable. It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower viscosity decreases shear stress on the vascular wall, engendering vasoconstriction and reduced functional capillary density [66]. Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. A large molecular dimension such as HbV can provide viscous fluids. In relation to this, our recent studies clarified that HbV suspended a series of plasma substitutes can provide non-Newtonian viscous fluid without capillary plugging [67]. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors. These new concepts suggest reconsideration of the design of artificial O_2 carriers [68]. Actually, new products are appearing, although they are in the preclinical stage, not only HbV, but also zero-link polymerized Hb [69] and others with larger molecular dimensions and higher O_2 affinities. Erni *et al.* clarified that HbV with a high O_2 affinity (low P_{50}, such as 9–15 torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischaemic skin flap [63, 70–72]. That study showed that HbV would retain O_2 in the upper arterioles, then perfuse through collateral arteries and deliver O_2 to the targeted ischaemic tissues. The results imply the further application of HbV for other ischaemic diseases such as myocardial and brain infarction and stroke.

Concluding remarks

Advantages of artificial O_2 carriers including HbV are the absence of blood-type antigens and infectious viruses, along with stability for a long-term storage for any emergency that might overwhelm the RBC transfusion capacity. The shorter half-lives of the HbV in the bloodstream (2–3 days) limit their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties of HbV supports the possible tailor-made O_2 carriers that suit various clinical indications. The achievements of ongoing HbV research described above make us confident in advancing further development of HbV, with the expectation of its eventual realization.

Conflicts of interest statement

Among the authors, ET, HS, KS and KK are consultants of Oxygenix Inc. (Tokyo, Japan).

Acknowledgements

This work was partly supported by Health Sciences Research Grants (Research on Regulatory Science), the Ministry of Health, Labour and Welfare, Japan. The authors gratefully acknowledge Prof. S. Takeoka (Waseda University); Prof. Yozu, Prof. Suematsu, Dr M. Watanabe, Dr Y. Izumi, Dr M. Yamamoto, Dr T. Ikeda, Dr E. Ikeda (Keio University); Dr H. Ikeda, Dr H. Azuma, Dr M. Fujiwara, Dr H. Abe (Hokkaido Red Cross Blood Center); Dr M. Takaori (Higashitakarazuka Satoh Hospital); Prof. M. Otagiri, Dr M. Anraku (Kumamoto University); Prof. N. Maeda, Dr Y. Suzuki (Ehime University); Prof. M. Intaglietta,

Dr A.G. Tsai, Dr P. Cabrales (University of California, San Diego); Prof. W.T. Phillips (University of Texas, San Antonio); Prof. D. Erni (Inselspital Hospital, University of Berne) and their active colleagues for meaningful discussions and contributions to this research.

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Artificial Cells, Blood Substitutes, and Biotechnology, 35: 157–172, 2007
Copyright © Informa Healthcare
ISSN: 1073-1199 print/1532-4184 online
DOI: 10.1080/10731190601188224

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Effects of Hemoglobin Vesicles, a Liposomal Artificial Oxygen Carrier, on Hematological Responses, Complement and Anaphylactic Reactions in Rats

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Abstract: Hemoglobin vesicle (HbV), a liposomal oxygen carrier containing human hemoglobin, was intravenously infused into rats. After the infusion of saline, the HbV or empty vesicle (EV), numbers of red cells, leukocytes and platelets in peripheral blood were unchanged during the observation period of one week in addition to each time point among three groups. However, the lymphocyte ratio transiently decreased and the granulocyte ratio increased in the HbV and EV groups at 6 h after the infusion. Those changes returned to the initial value one day after the infusion and those were maintained for the subsequent observation period. No dramatic change was seen in the ratio of $CD4^+/CD8^+$ T cells.

A transient decrease of the complement titer was observed three days after the infusion of HbV and EV, although the consumption of complement titer was not detected in rat serum by mixing HbV or EV *in vitro*, indicating that the transient decrease of complement titer *in vivo* was not due to the consumption of complement due to the interaction with HbV or EV. Multiple infusions of HbV caused the decrease of complement titer only after the first infusion and no allergic reaction was observed. No anaphylactic shock was observed in rats

This study was supported, in part, by a Health Science Research Grant (Artificial Blood Project) from the Ministry of Health and Welfare, Japan, and by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture.
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administered with EV several times, while ovalbumin (OVA) sensitized rats died with symptoms of respiratory distress after the second OVA administration. These results indicate that HbV could be administered without serious clinical symptoms or adverse reactions.

Keywords: Anaphylaxis; Complement; Hemoglobin vesicle; Hematological response; Liposome

INTRODUCTION

Artificial oxygen carriers as a red cell substitute have been developed for their emergency use, capacity for long-term storage, being no requirement of cross-match and pathogen-free blood components. Hemoglobin itself, however, is not applicable as an artificial oxygen carrier because it easily dissociates from a tetramer into a dimer and exhibits nephrotoxicity [1]. In addition, hemoglobin is considered to induce vasoconstriction based on the nitric oxide (NO) scavenging mechanism at a site between endothelium cells and the vascular smooth muscle layer [2,3]. Multiple approaches have been taken to modify Hb to resolve these limitations, including chemical crosslinking to prevent dissociation, and polymerization or polyethyleneglycol (PEG) conjugation to increase its molecular mass to avoid penetration into the endothelium gap junction [1,4–9]. Recently, recombinant hemoglobin that has a lower affinity for NO has been developed and showed the absence of vasoconstriction [10]. On the other hand, the potential use of microencapsulated-hemoglobin into polymer as a cellular type of artificial oxygen carrier was reported in 1964 [4]. Since then, the encapsulation of hemoglobin into a lipid bilayer (i.e. liposome) (liposome-encapsulated hemoglobin; LEH) to make a cellular type oxygen carrier has been developed, and this type of oxygen carrier has been regarded as another approach to prevent side-effects and toxicity of Hb [11–15].

The hemoglobin vesicle (HbV) is a human hemoglobin encapsulated into liposomes with a PEG surface modification. We have revealed that HbV exhibits less interaction with platelets [16,17], neutrophils [18] and plasma proteins including complement [19] *in vitro* using human blood. HbV was revealed not to activate and consume complement *in vitro* using human serum, while a similar type of artificial oxygen carrier, LEH, did activate the complement and triggered pseudoallergic reactions [20–22]. Therefore, it is of importance to observe whether HbV affects the complement titer *in vivo*. In addition, we wished to know whether repeated administration of HbV induces an allergic or anaphylactic reaction.

Several studies showed that HbV resuscitated against hemorrhagic shock equivalent to red cell transfusion in model rats [23,24] and rabbits

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[25]. HbV infusion into rats induced minor changes in aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase without deteriorative liver damage, a transient increase of cholesterol and phospholipids and reversible changes of amylase and lipase levels in the plasma [26]. Thus, HbV infusion transiently or reversibly affects biochemical parameters in the rat plasma.

In this study, we have investigated the effects of HbV on the hematological characteristics and complement titer using rats. In addition, the immune response after HbV infusion was also examined.

METHODS

HbV

HbV was prepared as previously described [27,28]. Briefly, hemoglobin solution prepared from out-dated red blood cells obtained from blood centers was heated under a CO gas atmosphere to inactivate any possibly contaminating viruses and to remove the stroma and non-hemoglobin proteins [29]. After the centrifugation and filtration, hemoglobin solution was mixed with lipids and then extruded through membrane filters with a pore size of 0.22 μm to make liposomes. The lipid composition (mol%) was as follows: dipalmitoyl phosphatidylcholine (DPPC):cholesterol (CHOL):dipalmitoyl-L-glutamate-*N*-succinic acid (DPEA):polyethylene glycol-conjugated distearoyl phosphatidylethanolamine (PEG5000-DSPE) = 5:5:1:0.033. The mean particle size was 250 nm. All lipids were purchased from Nippon Fine Chemical Co. (Osaka, Japan) except PEG5000-DSPE, which was from NOF Co. (Tokyo, Japan). HbV was suspended in saline and contained 10 g of hemoglobin/dl, 5.7 g lipids/dl and <0.1 endotoxin unit of lipopolysaccharide/ml. An empty vesicle (EV), which consisted of the same lipid composition as HbV without hemoglobin encapsulation, was also prepared.

Animals and HbV Infusion

WKAH male rats, 8–10 weeks old and weighing 220–300 g, were purchased from Japan SLC (Shizuoka, Japan). Under ether anesthesia, HbV or EV was intravenously infused into rats from the tail vein at top-load. As a control, saline was infused into the rats. The injection volume was 20% of the whole blood volume according to the estimation as follows that the whole blood volume is 56 ml per kg of body weight in rats. For the experiments investigating the anaphylactic reaction, Brown Norway male rats, 8 weeks old and weighing 200–220 g (Japan SLC),

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were used because of their highly sensitive behavior to allergic and anaphylactic reactions [30].

Hematological Analysis

Before and after the infusion of HbV, EV or saline, peripheral blood was collected from the tail vein into a plastic tube coated with EDTA. Numbers of red blood cells (RBC), white blood cells (WBC) and platelets (PLT) were measured using an automatic cell counter (AcT diff; Beckman Coulter, Miami, FL, USA). The leukocyte population was measured with a flow cytometer (EPICS XL; Beckman Coulter) based on forward-and side-scatter plot analysis. The lymphocyte subset was analyzed with the flow cytometer after staining with monoclonal antibodies; PE-labeled anti-CD4 (Beckman Coulter), FITC-labeled anti-CD8b (BD Biosciences, San Jose, CA, USA).

Complement Study

Before and after the infusion of HbV, EV or saline, peripheral blood was collected into the glass tube from the tail vein. Blood was clotted by standing for 1 h at room temperature and then for 1 h at 4°C. Serum was separated from the blood clot by centrifugation at 2,000 g for 20 min at 4°C, followed by additional centrifugation at 15,000 g for 45 min at 4°C to separate the serum from HbV or LE, and then stored at −80°C until assay.

In another complement experiment *in vivo*, HbV or saline was administered four times at two-day intervals from the first injection. Blood was collected before each injection and at subsequent time intervals. The serum was prepared as described above and stored at −80°C until assay.

In the *in vitro* study, rat serum was prepared from five non-treated rats. The serum was incubated with HbV, EV or saline at a ratio of 80:20 or 60:40 (v/v) at 37°C for 1 h. After centrifugation at 15,000 g for 45 min at 4°C, the supernatants were stored at −80°C until assay.

The complement titer was measured with a 50% hemolysis assay based on Mayer's method using a commercial kit (New One point CH50 (KW); Japan BCG Supply Co., Tokyo, Japan), which was approved to be applicable to the measurement of rat serum complement [31]. The unit of complement titer was expressed as CH50 (U/ml).

Examination of Anaphylactic Reaction

EV was used in this study to avoid interfering heterologous immune responses against human hemoglobin in HbV. Brown Norway rats were

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intravenously administered with EV (1–2 ml). The second infusion was carried out at 2 weeks, the third infusion was at 4 weeks, and the fourth infusion was at 8 weeks after the first infusion. As a positive control of the anaphylactic reaction model, Brown Norway rats were immunized subcutaneously with ovalbumin (OVA) in combination with the complete Freud's adjuvant. Two weeks after the immunization, OVA was intravenously infused from the tail vein. Clinical behavior, such as fluffiness, tear, blood drain speed and respiration, was observed during and after the infusion.

Statistical Analysis

Experimental differences from the controls (i.e. saline group) among the three groups were assessed with Non-repeated Measures ANOVA followed by the Dunnett test as a post-hoc test. The comparison of two groups was made with the Unpaired Student's t-test. A computer statistics package was utilized for statistical analysis (ystat2004, IGAKUTOSYO Press Co. Ltd., Tokyo, Japan). Values of $p < 0.05$ were considered significant.

RESULTS

Hematological Changes After HbV Infusion

Hematological markers were investigated at 6 h and 1, 3 and 7 days after the infusion of HbV, EV or saline. During the observation period, numbers of RBC, WBC and PLT varied in three groups. However, no significant differences were observed among the three groups at any time point (Fig. 1).

For the leukocyte population, the transient decrease of the lymphocyte ratio and the increase of the granulocyte ratio was significant in the HbV and EV groups at 6 h after the infusion. The 80.1% lymphocyte ratio in the control was decreased to 71.4% in EV and 56.7% in HbV. On the other hand, the 14.0% granulocyte ratio in the control was increased to 25.9% in EV and 40% in HbV. The degrees of the decrease and the increase were significantly greater in the HbV group than the EV group. The ratio of monocytes decreased 6 h after the infusion in three groups and significant differences were observed between the HbV and EV groups and the control group. The decrease of monocytes gradually returned to the initial level. The ratios varied from 2.7% to 10.4% (Fig. 2).

A subset of lymphocytes was analyzed by flow cytometry using monoclonal antibodies. Ratios of T cells and B cells were unchanged in all groups during the observation period (data not shown). As shown

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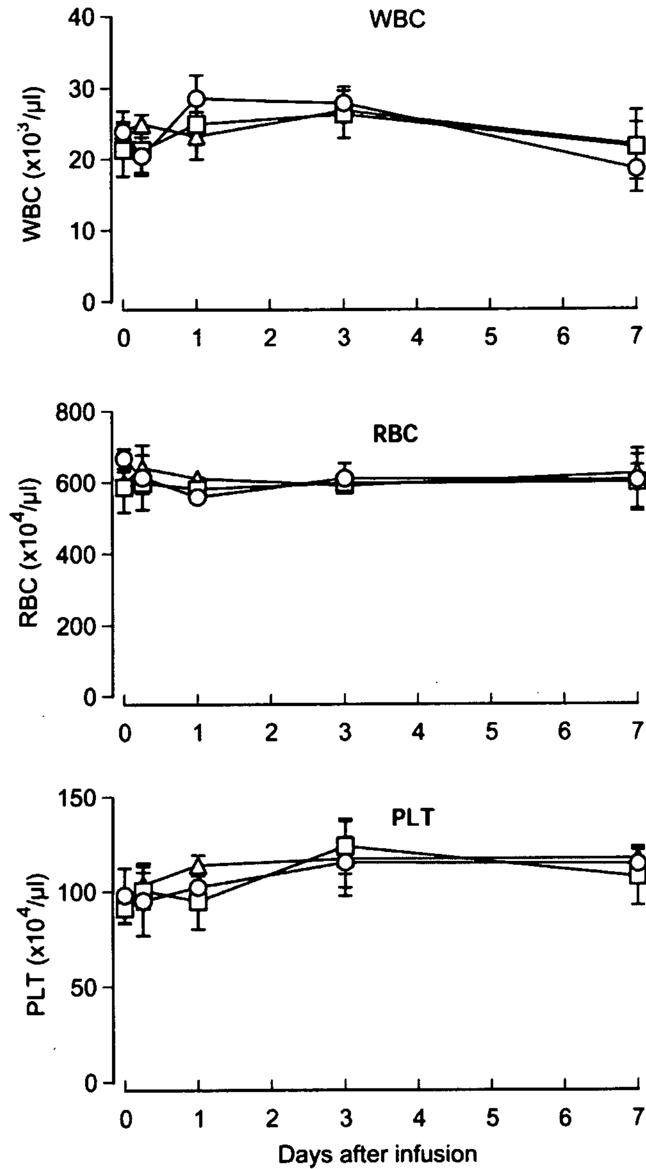

Figure 1. Effects of HbV infusion on peripheral blood cells. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and numbers of red cells, white cells and platelets were counted with an automatic cell counter. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean ± SD.

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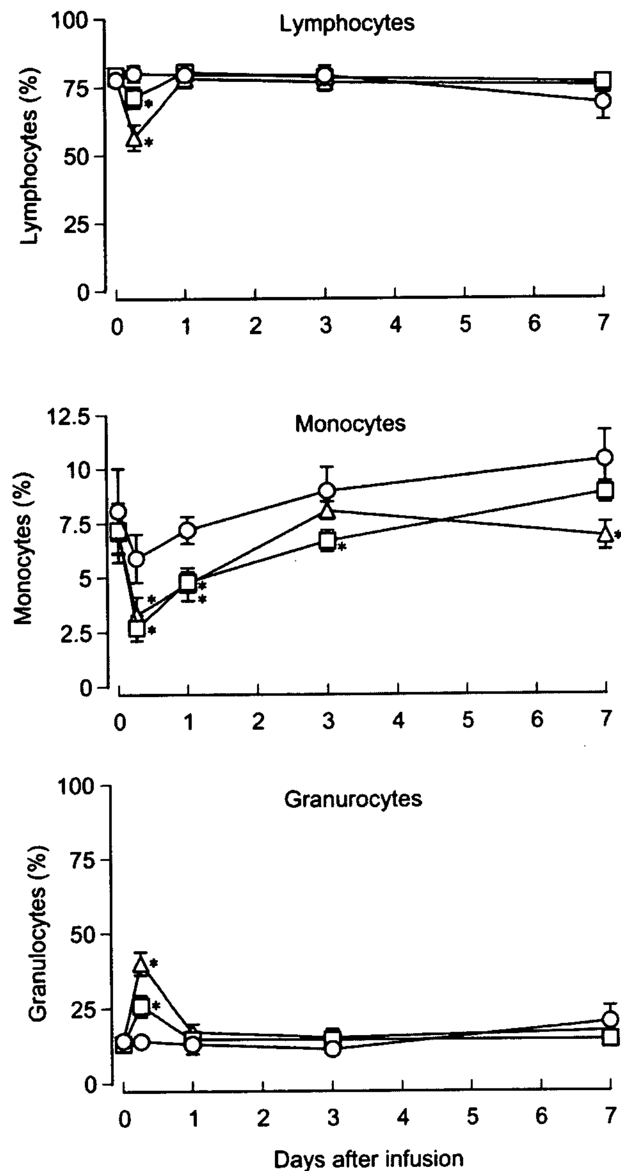

Figure 2. Effects of HbV infusion on the leukocyte population. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and ratios of lymphocytes, monocytes and granulocytes were analyzed with the flow cytometer. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean ± SD. $^*p < 0.05$.

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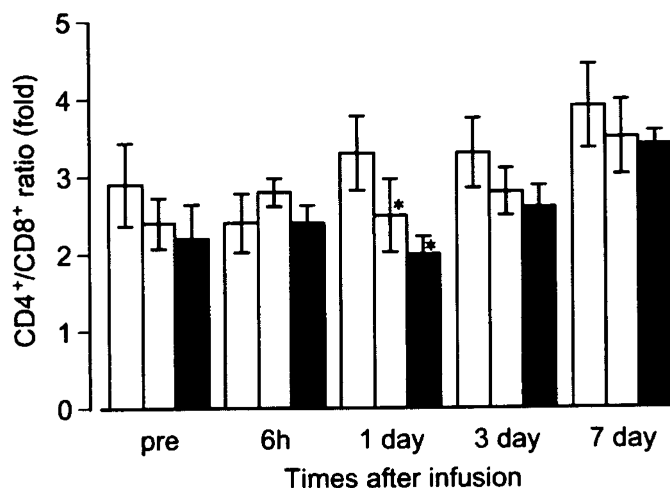

Figure 3. Effects of HbV infusion on the T cell subset. HbV, EV or saline was infused into rats at top-load from the tail vein. Blood was sampled and ratios of $CD4^+$ or $CD8^+$ T cells were analyzed as described in the Materials and Methods section. Black bars, HbV; gray bars, EV; white bars, saline. N = 3–4, mean ± SD. $^*p < 0.05$.

in Figure 3, ratios of $CD4^+/CD8^+$ T cells varied from 2.0 to 3.9 of the mean value in the series of experiments. However, statistical significance was not observed in the HbV and EV groups versus control group except on day 1. The mean values of the $CD4^+/CD8^+$ ratios in the HbV and EV groups were generally lower than those of the control group.

Effects of HbV on the Complement Titer

The complement titer in rat serum was assessed before and after the infusion of HbV, EV or saline. As shown in Figure 4, the complement titer dropped significantly three days after the infusion of HbV or EV, then afterwards it gradually returned to the levels before infusion and of the saline-infused group on day 14. No significant difference was observed between the HbV-infused group and the EV-infused group regarding the degree of decrease.

HbV, EV or saline was mixed with rat serum *in vitro* at a ratio of 20:80 or 40:60 and incubated for 1 h at 37°C. The complement titer decreased in accordance to the dilution of serum by mixing HbV, EV or saline (Figure 5). No significant difference was observed among three subjects at any mixing ratios, indicating that both HbV and EV did not consume complement.

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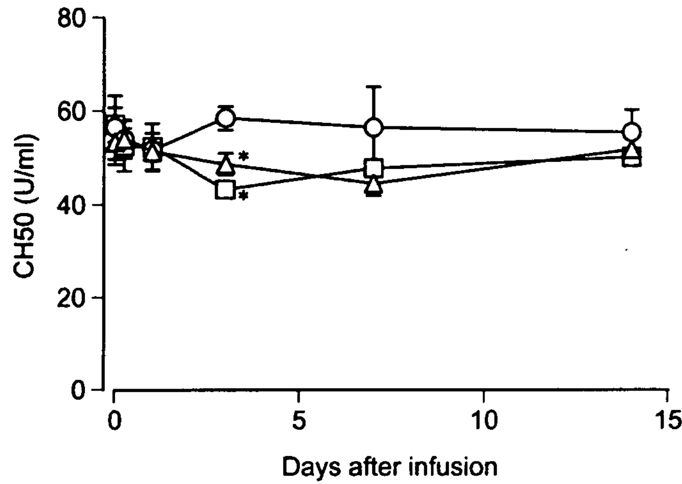

Figure 4. Changes of the complement titer in the rat serum after HbV infusion. HbV, EV or saline was infused into the rats at top-load from the tail vein. Blood was sampled, and sera were prepared as described in the Materials and Methods section. The complement titer was measured and indicated as CH50. Triangles, HbV; squares, EV; circles, saline. N = 3–4, mean ± SD. *$p < 0.05$.

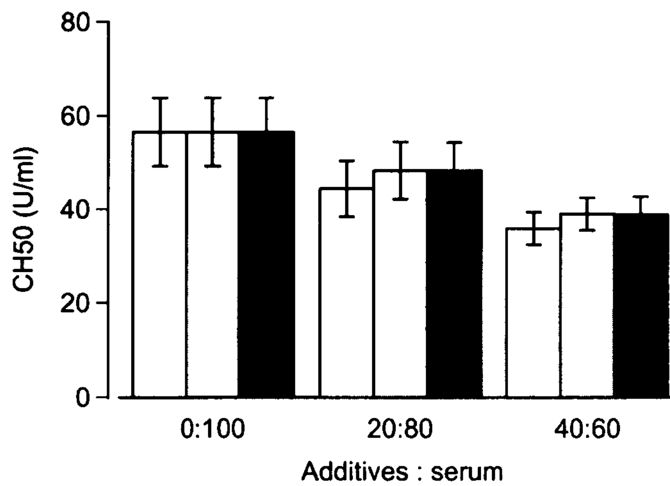

Figure 5. Effects of HbV on the rat complement *in vitro*. HbV, EV or saline was mixed with rat sera and incubated at 37°C for 1 hr. After centrifugation, the complement titer in the supernatant was measured. Black bars, HbV; gray bars, EV; white bars, saline. N = 5, mean±SD.

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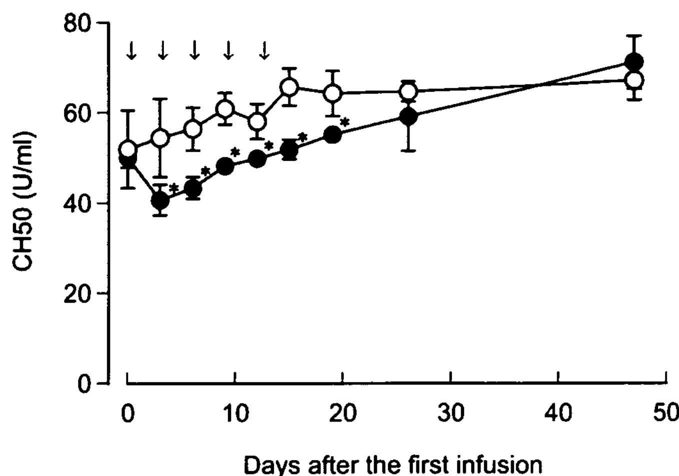

Figure 6. Effects of repeated infusion of HbV on the rat serum complement. HbV or saline was infused into rats at top-load from the tail vein at the time point as indicated by arrows. Blood sampling, sera preparation and complement measurement were carried out as described in the Materials and Methods section. Closed circles, HbV; open circles, saline. N = 3–5, mean ± SD. $^{*}p<0.05$.

HbV was sequentially infused five times with two-day intervals. The complement titer dropped on day 3 after the first injection (Figure 6). However, the additional infusion of HbV with 2-day intervals no longer further decreased the complement titer. Two weeks after the final injection, on day 26, no significant difference was observed in the complement titer between two groups.

Assessment of Anaphylactic Reaction Caused by EV

Because HbV contains human hemoglobin, which may act as an antigen in rats, EV was used to assess the potential to induce anaphylactic reaction caused by liposome components. No abnormal behavior, such as fluffiness and tear, was observed in the EV-infused rats and the respiration was kept normal. All animals were alive in the EV-infused group even after the final injection with symptomless (Table 1). OVA administration was performed to evaluate whether this rat model is appropriate to assess anaphylactic reaction. Rats were sensitized subcutaneously with OVA using complete Freud's adjuvant to obtain complete sensitization. Two weeks after the sensitization, OVA was intravenously infused from the tail vein. Immediately after the infusion, all rats were affected with respiratory distress and the blood drain speed dropped, resulting in death.