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表　研究成果の刊行に関する一覧表

刊行書籍又は雑誌名（雑誌のときは雑誌名、巻号数、論文名）	刊行年月日	刊行書店名	執筆者名
Phagocytosis of liposome particles by rat splenic immature monocytes makes them transiently and highly immunosuppressive. *J. Pharmacol. Exp. Therap.* 337, 42-49 (2011).	2011 年 4 月	The American Society for Pharmacology and Experimental Therapeutics	D. Takahashi, H. Azuma, H. Sakai, K. Sou, D. Wakita, H. Abe, M. Fujihara, H. Horinouchi, K. Kobayashi, T. Nishimura, H. Ikeda.
Fluid resuscitation with hemoglobin-vesicles prevents Esherichia coli growth via complement activation in a hemorrhagic shock rat model. *J. Pharmacol. Exp. Therap.* 337, 201-208 (2011)	2011 年 4 月	The American Society for Pharmacology and Experimental Therapeutics	K. Taguchi, S. Ogaki, H. Watanabe, D. Kadowaki, H. Sakai, K. Kobayashi, H. Horinouchi, T. Maruyama, M. Otagiri.
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Hemoglobin vesicle improves recovery of cardiac function after ischemia-reperfusion by attenuating oxidative stress in isolated rat heart. *J. Cardiovasc. Pharmacol.* 58, 528-534 (2011).	2011 年 11 月	Lippincott Williams & Wilkins	J. Nakajima, M. Bessho, T. Adachi, T. Yamagishi, S. Tokuno, H. Horinouchi, F. Ohsuzu.
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Fluid resuscitation of hemorrhagic shock with Hemoglobin vesicles in Beagle dogs: pilot study. *Artif. Cells Blood Substitutes Biotechnol.* 40, 179-195 (**2012**)	2012 年 2 月	Informa Healthcare	M. Yamamoto, H. Horinouchi, Y. Seishi, N. Sato, M. Itoh, K. Kobayashi, H. Sakai
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A new method for measuring the oxygen diffusion constant and oxygen consumption rate of arteriolar walls. *Keio J. Med.* (in press)	印刷中	慶應義塾大学医学部出版会	Sasaki　N, Horinouchi H, Ushiyama A, Minamiani H.
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Phagocytosis of Liposome Particles by Rat Splenic Immature Monocytes Makes Them Transiently and Highly Immunosuppressive In Ex Vivo Culture Conditions

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ABSTRACT

Liposomes reportedly accumulate in monophagocytic systems (MPSs), such as those of the spleen. Accumulation of considerable amounts of liposome in a MPS can affect immunologic response. While developing a liposomal oxygen carrier containing human hemoglobin vesicle (HbV), we identified its suppressive effect on the proliferation of rat splenic T cells. The aim of this study was to elucidate the mechanism underlying that phenomenon and its effect on both local and systemic immune response. For this study, we infused HbV intravenously at a volume of 20% of whole blood or empty liposomes into rats, removed their spleens, and evaluated T cell responses to concanavalin A (Con A) or keyhole limpet hemocyanin (KLH) by measuring the amount of [^{3}H]thymidine incorporated into DNA. Cells that phagocytized liposomal particles were sorted using flow cytometry and analyzed. Serum anti-KLH antibody was measured after immunizing rats with KLH. Results showed that T cell proliferation in response to Con A or KLH was inhibited from 6 h to 3 days after the liposome injection. Direct cell-to-cell contact was necessary for the suppression. Both inducible nitric-oxide synthase and arginase inhibitors restored T cell proliferation to some degree. The suppression abated 7 days later. Cells that trapped vesicles were responsible for the suppression. Most expressed CD11b/c but lacked class II molecules. However, the primary antibody response to KLH was unaffected. We conclude that the phagocytosis of the large load of liposomal particles by rat CD11b/c+, class II immature monocytes temporarily renders them highly immunosuppressive, but the systemic immune response was unaffected.

Introduction

A liposome is a lipid particle that is widely used as a drug vehicle in clinical settings and as an adjuvant or delivery system for vaccine antigens. For example, the delivery of Ag inside lipid vesicles is expected to enhance its uptake by antigen-presenting cells such as macrophages and dendritic cells (Dal Monte and Szoka, 1989; Guéry et al., 1996; Dupuis et al., 2001), thereby augmenting the immune response. However, some macrophages act as immune suppressor cells (suppressor macrophages) under certain pathological conditions; the production of nitric oxide (NO) is reportedly involved in that suppression activity (Albina et al., 1991; al-Ramadi et al., 1991; Schleifer and Mansfield, 1993; Dasgupta et al., 1999).

The monophagocytic system (MPS) includes various cells, monocytes, and macrophages capable of phagocytizing particles (Randolph et al., 1999; Dupuis et al., 2001). Because liposomes are particulate, they accumulate in the MPS pres-

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ABBREVIATIONS: NO, nitric oxide; iNOS, inducible NO synthase; MPS, monophagocytic system; HbV, hemoglobin vesicle; EV, empty vesicle; KLH, keyhole limpet hemocyanin; DPPC, dipalmitoyl phosphatidylcholine; CHOL, cholesterol; DHSG, 1,5-*O*- dihexadecyl-*N*-succinyl-L-glutamate; L-NMMA, N^G-monomethyl-L-arginine; nor-NOHA, *N*-ω-hydroxy-nor-L-arginine; CFSE, carboxyfluorescein diacetate succinimidyl ester; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FCM, flow cytometry; Con A, concanavalin A; PEG, polyethylene glycol; DSPE, 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine; PHA-M, phytohemagglutinin M; IL, interleukin; PBS, phosphate-buffered saline.

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ent in the liver, spleen, bone marrow, and other tissues when injected intravenously into experimental animals (Torchilin, 2005). Therefore, accumulation of liposome in monocyte/macrophages can negatively affect local immune function. Nevertheless, no such effect has been reported to date, possibly because the amount of infused liposome is usually small.

Artificial red blood cells (artificial oxygen carriers) are classifiable into two major types: cell-free and cellular (Ajisaka and Iwashita, 1980; DeVenuto and Zegna, 1983; Chatterjee et al., 1986; Natanson et al., 2008). The latter include hemoglobin molecules encapsulated by liposomes, the major component of which is dipalmitoyl phosphatidylcholine (DPPC) and designated as hemoglobin vesicles (HbVs) (Djordjevich and Miller, 1980; Sakai et al., 1997; Phillips et al., 1999; Chang, 2004). In fact, HbVs have functioned well as blood substitutes in animal models with no noteworthy adverse reactions either in vivo (Sakai et al., 2004; Yoshizu et al., 2004; Cabrales et al., 2005) or in vitro (Ito et al., 2001; Wakamoto et al., 2005; Abe et al., 2006). They also reportedly accumulate in components of the MPS soon after their administration (Sou et al., 2005).

The amount of HbV to be infused as a blood substitute is quite large. Therefore, the negative effect of the liposome on immunological functions might be amplified and easily detected.

In fact, we recently found splenic T cell proliferation to be temporarily but dramatically suppressed after massive administration of HbV into rats. Considering that many trials of cancer immunotherapy have used liposomes containing DPPC, the mechanism behind this immune suppression should be elucidated. In this study, we identified the cells responsible for this phenomenon, elucidated the mechanism behind it, and assessed its effect on both local and systemic immune response. These results might contribute to the progress not only of a liposome-based cancer vaccine strategy but also to progressive development of artificial oxygen carriers such as HbVs.

Materials and Methods

Preparation of HbV and Liposome Suspension. The HbVs were prepared as described previously (Sakai et al., 1997; Sou et al., 2003). In brief, a hemoglobin solution prepared from outdated red blood cells was heated under a CO gas atmosphere to inactivate any contaminating virus (Abe et al., 2001). After centrifugation and filtration, the hemoglobin solution was mixed with lipids and then extruded through membrane filters with a 0.22-μm pore size to produce liposomes. The lipid composition (molar ratio) was as follows: 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC)/cholesterol (CHOL)/1,5-*O*- dihexadecyl-*N*-succinyl-L-glutamate (DHSG)/polyethylene glycol-conjugated 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (PEG_{5000}-DSPE) = 5:5:1:0.033. The mean particle size was 250 nm. All lipids were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan) except PEG_{5000}-DSPE (NOF Corp., Tokyo, Japan). The HbVs were suspended in normal saline; the suspension contained 10 g of hemoglobin/dl, 5.7 g of lipid/dl, and <0.1 endotoxin unit of lipopolysaccharide/ml. Empty vesicles (EVs), which consisted of the same lipid composition as HbV without hemoglobin encapsulation, were also prepared. Liposomes that were composed solely of DPPC (designated DPPC-liposomes) were prepared.

Preparation of FITC-Labeled Empty Vesicle (FITC-Liposome). Using mixed lipids of DPPC, CHOL, DHSG, and PEG-DSPE (5:5:1:0.033, molar ratio) including 0.1 mol% of *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine and triethylammonium salt (FITC-lipid; Molecular Probes, Carlsbad, CA), FITC-labeled empty vesicles, designated FITC-liposome, were prepared. The mixed lipid powder was hydrated with saline solution at 7 g/dL. The dispersion was introduced into an extruder (Lipex Biomembrane Inc., Vancouver, Canada) and extruded through the membrane filters (final pore size, 0.22 μm, Durapore; Nihon Millipore Ltd., Tokyo, Japan) under pressure using nitrogen gas to obtain FITC-liposome with 233-nm mean diameter.

Animals and Injection of HbV (or Other Liposomes). Male WKAH rats, 8 to 12 weeks old and weighing 220 to 300 g, were purchased from Japan SLC Inc. (Shizuoka, Japan). Under ether anesthesia, HbVs (or other liposomes) were infused intravenously into the tail at the top load. Saline was infused as a control. The injection volume was 20% of whole blood volume, which was estimated as 56 $ml \cdot kg^{-1}$ of body weight in rats. This is consistent with approximately 1 liter of whole blood in a 70-kg male human, an amount that is likely to occur in a clinical setting. After the injection, the spleen or lymph node was excised aseptically under ether anesthesia. Then, a single-cell suspension was prepared. Erythrocytes were depleted using red blood cell lysing buffer (IBL, Hamburg, Germany). Then spleen cells were washed in RPMI medium 1640 containing 10% fetal calf serum (FCS). The Executive Review Board (functioning as an Institutional Review Board) of the Hokkaido Red Cross Blood Center reviewed and approved this study's protocol.

Preimmunization with Keyhole Limpet Hemocyanin. To generate keyhole limpet hemocyanin (KLH)-specific T cells, KLH solution (200 μg of KLH in 0.5 ml of saline) or saline was mixed with the same volume of incomplete Freund's adjuvant. Then 0.5 ml of the mixture (100 μg of KLH) was injected subcutaneously. The HbV or saline was injected 7 days after injection of KLH because preliminary experiment showed that induction of KLH-specific T cell response in the spleen was achieved by 7 days after injection of KLH. Subsequently, the spleen was excised at 6 h, 1 day, 3 days, and 7 days, and the proliferative response of KLH-specific splenic T cells was assayed in vitro.

Assay of the Proliferation of Splenic T Cells in Response to Con A or KLH. Single spleen cell suspensions in RPMI medium 1640 supplemented with 10% FCS and mercaptoethanol (50 μM) were plated in 96-well plates in a volume of 0.2 ml/well. The cells were cultured in triplicate for 72 h in the presence of 0.3 and 3 μg/ml of Con A (Sigma-Aldrich, St. Louis, MO) or KLH (30 μg/ml) and pulsed with [^{3}H]thymidine (18.5 kBq) for the final 18 h of incubation. Phytohemagglutinin M (PHA-M) was also used as a T cell mitogen in some experiments. Subsequently, the cells were harvested onto glass fiber paper. Radioactivity was measured using a liquid scintillation counter (LS5000 TD; Beckman Coulter, Fullerton, CA). For some experiments, control spleen cells (1×10^5) were mixed with HbV or EV-loaded spleen cells (1×10^5), which were taken from rat loaded with HbV or EV 24 h before, and plated in 96-well plates in a volume of 0.2 ml/well.

Assay of Nitric Oxide and IL-2 Production. The production of NO in the culture supernatant after 48-h incubation in the presence of Con A (0.3 μg/ml) was measured as the concentration of nitrite using a Griess Assay kit (R&D Systems, Minneapolis, MN). The amount of IL-2 was also measured using the rat IL-2 Immunoassay Quantikine kit (R&D Systems).

Evaluation of T Cell-Suppressive Effect of Liposome-Phagocytized Cells. Cells that were positive for FITC were sorted from FITC-liposome-loaded splenocytes using FCM (Aria; BD Biosciences, San Jose, CA). Their suppressive effect on the proliferation of Con A-stimulated bulk splenocytes was assayed.

Analysis of Cell Surface Markers and Cell Sorting. Cell surface markers were analyzed using an LSR flow cytometer (BD Biosciences). The antibodies used for the analysis were allophycocyanin-conjugated CD11b/c (OX42) and phycoerythrin-conjugated anti-class II (OX6), CD80, CD86, CD8a (OX8), CD25 (OX39), CD11a (WT-1), CD172 (OX41), HIS48 (anti-granulocytes), CD103 (OX62), and anti-

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CD161 (NKR-P1A), and FITC-conjugated anti-rat CD25, all of which were purchased from BD Biosciences. In addition, allophycocyanin-conjugated anti-rat CD3 was purchased from Immunotech (Marseille, France). For each analysis, at least 10,000 events were collected and analyzed using Cellquest software (BD Biosciences).

Detection of Cells with Intracytoplasmic iNOS Protein. To detect intracytoplasmic inducible NO synthase (iNOS), cells were fixed using FACS Lysing Solution (BD Biosciences) and permeabilized using PBS containing 0.1% saponin and 1% FCS. Then they were stained with mouse FITC-conjugated anti-rat CD11b (BD Biosciences) and phycoerythrin-conjugated anti-rat NOS2 (BioLegend, San Diego, CA).

Histological Staining. In some experiments, splenocytes were spun on slides with Cytospin (Thermo Fisher Scientific, Waltham, MA) and stained with May-Grunwald-Giemsa dye (Merck, Darmstadt, Germany). Alternatively, the spleen was fixed with formalin and stained with hematoxylin (Sigma-Aldrich) and eosin (Merck Diagnostica, West Point, PA), respectively, then observed under a light microscope (BX50; Olympus, Tokyo, Japan). Microscopic images were captured using a digital camera (MP5Mc/OL; Olympus) and processed using Win Roof ver. 5.5 software (Mitani Corp., Tokyo, Japan).

Evaluation of the Effect of L-NMMA and nor-NOHA on the Suppression of Splenocyte Proliferation. For some experiments, an iNOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA) (2 mM; Alexis Corp., San Diego, CA), or arginase inhibitor, *N*-ω-hydroxy-nor-L-arginine (nor-NOHA) (0.5 mM; Calbiochem, San Diego, CA), or both were added to the culture at final concentrations of 2 and 0.5 mM, respectively.

Transwell Experiment. Control bulk splenocytes were washed twice with 1% FCS/PBS and resuspended in the same solution at a concentration of 1×10^7/ml. Subsequently, the cells were incubated with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) at 37°C for 5 min; they were then washed and resuspended in 10% FCS/RPMI medium 1640. The HbV-loaded splenocytes (4×10^5) were placed in the upper chamber (Falcon Transwell, 0.4-μm pore size; BD Biosciences), and CFSE-stained control splenocytes (4×10^5) were placed in the lower chamber (Falcon culture plate). Then they were incubated in the presence of Con A (0.3 μg/ml). At the same time, only the control splenocytes (4×10^5) or a mixture of both vesicle-loaded and control splenocytes were cultured in the lower chamber in the presence of Con A (0.3 μg/ml). The fluorescence intensity of CFSE-stained cells was analyzed using FCM 72 h later.

Evaluation of the Effect of HbV on Primary Antibody Response. Rats were infused with HbV or saline 6 h before the intravenous injection of KLH (100 μg) on day 0. Peripheral blood was taken from the tail vein on days 5, 7, 10, and 14. Subsequently, serum was separated and stored at −80°C until anti-KLH antibody was measured.

The serum concentration of anti-KLH IgG was measured via enzyme immunoassay. Ninety-six-well microtiter plates (Nalge Nunc International, Rochester, NY) were coated with 0.5 μg of KLH in 100 μl of PBS per well and incubated overnight at 4°C. Plates were washed with PBS once and blocked with 5% dry skim milk in PBS. After incubation for 2 h at room temperature, plates were washed three times with PBS-0.1% polyoxyethylene sorbitan monolaurate (Tween 20). Rat sera were added at a concentration of 1:1000 40% FCS/0.05% Tween 20/PBS. Appropriately diluted standard rat anti-KLH IgG2a (BD Biosciences) in 40% FCS/0.05% Tween 20/PBS was also added to the appropriate plates. Plates were incubated for 60 min and washed three times. Then 100 μl of horseradish peroxidase-conjugated goat anti-rat IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added at a concentration of 1:10,000 in 0.05% skim milk/0.1% Tween 20/PBS to the appropriate plates and incubated for 60 min. Plates were washed three times, then 100 μl of the 3,3′,5,5′,-tetramethylbenzidine one-step substrate system (Dako Japan, Inc., Tokyo, Japan) was added to all wells. Plates were incubated for 15 min and read at an optical density of 450 nm.

Statistical Analyses. Experimental differences from the controls were assessed using two-sided unpaired Student's *t* tests. Software was used for statistical analyses (ystat2004; Igaku Tosho Press, Co. Ltd., Tokyo, Japan). Values of $p < 0.05$ were inferred as significant.

Results

Both HbV and Empty Vesicles Suppressed the Proliferative Response of Splenic T Cells to Con A. The proliferative response of both EV- and HbV-loaded splenic T cells was clearly inhibited at a lower dose of Con A (0.3 μg/ml) than that of saline-loaded (control) splenic T cells ($p < 0.01$) (Fig. 1a). This result was quite reproducible. However, at a high dose of Con A (3.0 μg/ml), the inhibition was mild. Therefore, the concentration of Con A used in the subsequent experiment was fixed at 0.3 μg/ml. As with splenocytes, the Con A-induced proliferation of HbV-loaded lymph node cells was also decreased at 0.3 μg/ml of Con A ($p < 0.05$) compared with that of control lymph node cells (Fig. 1b). In addition, the suppressive effect of EV tended to be stronger than that of HbV. Furthermore, PHA-M induced T cell proliferation was suppressed at 2 to 8 μg/ml of PHA-M (data not shown).

Kinetics of the HbV-Induced Suppression of the T Cell Proliferative Response to Con A and KLH. Suppression was observed clearly from 6 h to 3 days after the injection. It disappeared completely 7 days after the injection (Fig. 1c). The KLH-specific proliferation of splenic T cells was also inhibited by the HbV infusion, the kinetics being the same as that for Con A stimulation.

IL-2 and NO Were Detected in the Supernatant Cultured for 48 h in the Presence of Con A. Although the T cell proliferation of HbV-loaded splenocytes was inhibited reproducibly at 0.3 μg/ml of Con A, the amount of IL-2 in the supernatant of these splenocytes was comparable with that produced by saline-loaded splenocytes (Fig. 2a). Moreover, flow cytometric analysis revealed that the expression of high-affinity IL-2 receptor on the surface of the former T cells was comparable with that of the latter (data not shown). These data suggest that T cells were activated normally. Furthermore, NO was shown to be produced by both HbV-loaded and saline-loaded splenocytes, irrespective of the presence of Con A. However, the production of NO in HbV-loaded splenocyte culture supernatant tended to be higher than that of saline-loaded splenocyte culture supernatant (Fig. 2b).

T Cell Proliferation Was Restored by L-NMMA or nor-NOHA. Through the preliminary experiment, we confirmed that L-NMMA (iNOS inhibitor) (2 mM) and nor-NOHA (arginase inhibitor) (0.5 mM) used in the experiment did not influence the proliferation of Con A-stimulated saline-loaded T cells at all. Actually, L-NMMA(2 mM) was sufficient to inhibit the production of NO produced by Con A (0.3 μg/ml) stimulation, although nor-NOHA did not influence the production of NO at all (Fig. 2b). Both L-NMMA and nor-NOHA restored T cell proliferation to some degree, with better restoration by L-NMMA (Fig. 2c). These data show that NO was involved in the suppression of T cell proliferation.

Cell-to-Cell Contact Is Necessary for Suppression. When CFSE-stained control splenocytes were stimulated with Con A in the presence of HbV-loaded splenocytes, no cell division was observed (Fig. 2d, bottom). In contrast, when

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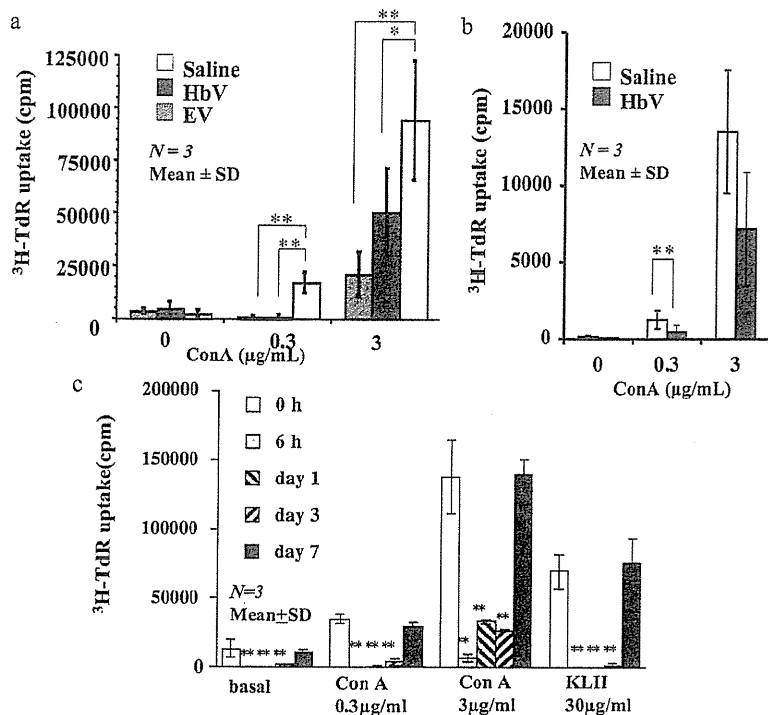

Fig. 1. Effect of HbV and empty vesicles on proliferation of Con A-stimulated rat splenic T cells. a, spleens were excised from rats 24 h after injection of HbV, EV, or saline (control); splenic single cells were stimulated with Con A. Both HbV and EV induced significant suppression of the proliferative response to Con A compared with saline. Data from three independent experiments were collected and expressed as mean ± S.D. b, abdominal lymph nodes were excised 24 h after injection of HbV. The lymph node single cells were stimulated with Con A. Proliferation of HbV-loaded lymph node cells was less than that of control lymph node cells at 0.3 μg/ml of Con A. Data are representative of two independent experiments. c, rats were immunized with KLH. After 7 days, they were injected with HbV. Spleens were excised 6 h, 1 day, 3 days, and 7 days later; the bulk splenocytes were stimulated with Con A or KLH. The proliferative response of splenic T cells to Con A and KLH was inhibited from 6 h to 3 days after injection of HbV. No suppression was observed after 7 days. Data are representative of at least three independent experiments and are expressed as mean ± S.D. **, $p < 0.01$ compared with control. *, $p < 0.05$ compared with control or day 7.

they were separated from HbV-loaded splenocytes using a transwell, considerable cell division was observed (Fig. 2d, middle).

Identification of Cells Responsible for T Cell Suppression. When control spleen cells (1×10^5) were stimulated using Con A (0.3 μg/ml) in the presence of spleen cells (1×10^5) loaded with HbV (or EV) 24 h prior, T cell proliferation was suppressed (Fig. 3a). Furthermore, cells that had phagocytized FITC-liposomes (FITC-positive cells) (Fig. 3b) inhibited the proliferation of control splenic T cells dose-dependently (Fig. 3c). In addition, FITC-negative cells proliferated well compared with FITC-EV-loaded bulk splenocytes. They proliferated even better than control splenocytes at 1 μg/ml of Con A (Fig. 3d).

Phenotypic Analysis of Cells Responsible for T Cell Suppression. The FITC-positive cells were gated first. These cells accounted for approximately 5% of all splenocytes. Then, the phenotype of those cells was analyzed. Most were positive for CD11b/c, but negative for class II molecules, CD80, or CD86 (Fig. 4a, top). In addition, they were weakly positive for CD4 and negative for CD3, CD25, CD8, and CD103. Some (14%) were positive for both CD11b/c and HIS48 (data not shown).

Microscopic examination revealed that unique cells appeared after the injection of vesicles. They were larger than lymphocytes. Moreover, they had cytoplasm that was abundant in vesicle-like particles and had a nucleus with an irregular rim (Fig. 4b).

Flow cytometric analyses of iNOS-positive cells revealed that the percentage of iNOS-positive cells among CD11b-positive cells is not necessarily higher in HbV-loaded splenocytes than in saline-loaded splenocytes (data not shown), suggesting HbV load does not increase in the number of NO-producing phagocytes.

Effect of HbV Infusion on Primary Antibody Response. Elevation of anti-KLH IgG antibody after the first challenge of KLH was demonstrated in both HbV-loaded and control rats, with no apparent difference between them (Fig. 5).

DPPC-Liposomes Can Induce Immune Suppression. Macroscopic examination revealed that, like HbV and empty vesicles, DPPC-liposome accumulated in the spleen (Fig. 6a). Subsequent histological examination revealed unique cells with abundant cytoplasm (Fig. 6b). Moreover, DPPC-liposomes induced suppression of T cell proliferation (Fig. 6c).

Discussion

We showed that the infusion of HbV temporarily suppressed not only mitogen-induced but also antigen-induced T cell proliferation in rat splenocytes (Fig. 1, a and c), which indicated that HbV infusion suppressed T cell receptor-mediated T cell proliferation.

The Hb molecule can be ruled out as a cause of suppression because even empty vesicles, HbV without Hb molecules in them, induced suppression.

Among the components of HbV, DPPC was responsible for the suppression. Similarly to HbV and EV, DPPC-liposomes, which contain no CHOL, DHSG, PEG, or Hb, were shown to accumulate in the spleen and induce suppression (Fig. 6). Whether CHOL, DHSG, and PEG are involved in the suppression or not remains unknown at present. However, considering that the suppressive effect of DPPC-liposome is more prominent than that of HbV, it is less likely that these components play a principal role in the suppression.

According to a previous report (Sakai et al., 2001), HbV began to accumulate in monocyte/macrophages present in the red pulp of the spleen. Then they were observed in considerable amounts on day 3, before gradually disappearing by

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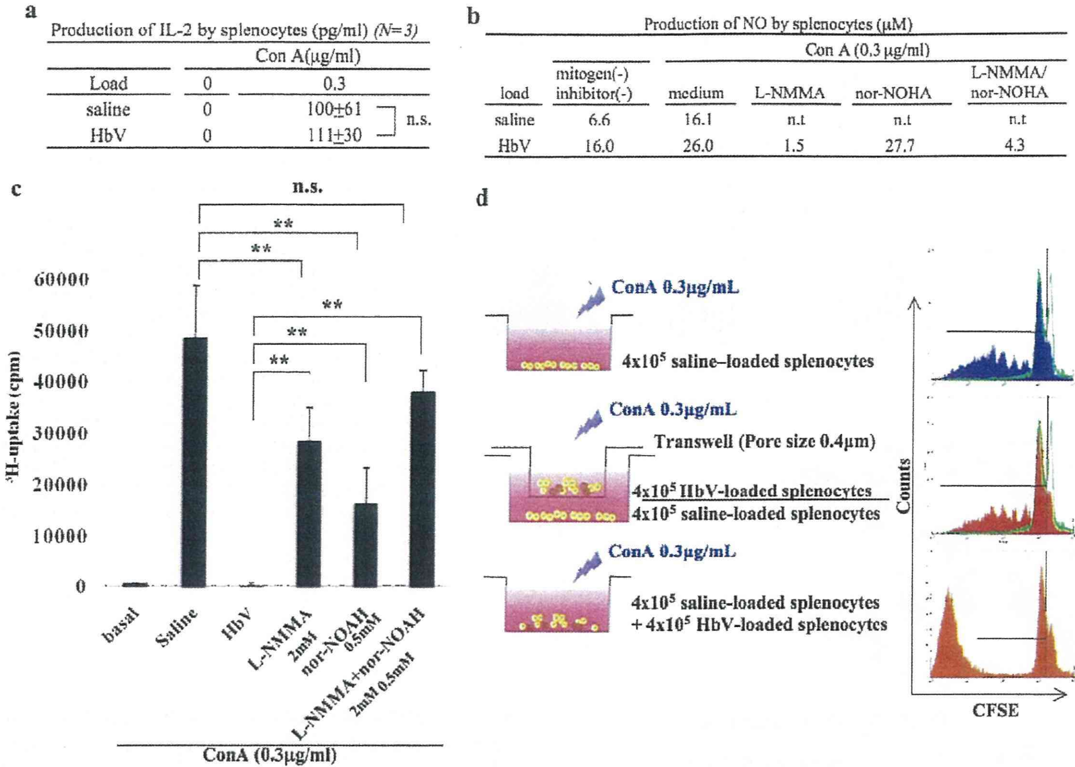


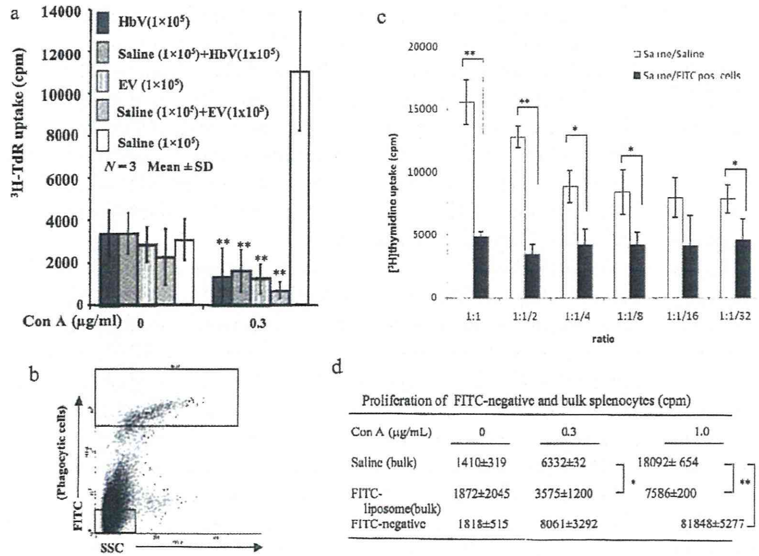
a

Production of IL-2 by splenocytes (pg/ml) (*N*=3)		
	Con A(μg/ml)	
Load	0	0.3
saline	0	100±61 (n.s.)
HbV	0	111±30

b

Production of NO by splenocytes (μM)					
		Con A (0.3 μg/ml)			
load	mitogen(-) inhibitor(-)	medium	L-NMMA	nor-NOHA	L-NMMA/ nor-NOHA
saline	6.6	16.1	n.t	n.t	n.t
HbV	16.0	26.0	1.5	27.7	4.3

Fig. 2. Analysis of the mechanism of T cell suppression. a, each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) for 48 h; then supernatant was collected and IL-2 was measured as described under *Materials and Methods*. Data were collected from three independent experiments and expressed as mean ± S.D. ($n = 3$). n.s., not significant ($p > 0.05$). b, splenocyte culture was performed in the presence or absence of L-NMMA and/or nor-NOHA for 48 h, then supernatant was collected and NO was measured as described under *Materials and Methods*. Data are representative of at least three independent experiments. c, each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) in the presence or absence of iNOS inhibitor (L-NMMA, 2 mM) or arginase inhibitor (nor-NOHA, 0.5 mM) or both. T cell proliferation was restored to a certain degree in the presence of each inhibitor. Significant inhibition disappeared in the presence of both inhibitors, suggesting that both iNOS and arginase were involved in the suppression. Data from two independent experiments are collected and expressed as mean ± S.D. ($n = 5$). **, $p < 0.01$. n.s., not significant. d, control bulk splenocytes were stained in advance with CFSE. Transwell chambers were used to prevent direct cell-to-cell contact between HbV-loaded bulk splenocytes (4×10^5) and control bulk splenocytes (4×10^5). Cell division was assessed by measuring the relative abundance of CFSE high and CFSE low T cells using FCM. Inhibition of cell division was observed only when they were mixed and cultured together. Data are representative of at least three independent experiments.



d

Proliferation of FITC-negative and bulk splenocytes (cpm)			
Con A (μg/mL)	0	0.3	1.0
Saline (bulk)	1410±319	6332±32	18092± 654
FITC-liposome(bulk)	1872±2045	3575±1200 *	7586±200 **
FITC-negative	1818±515	8061±3292	81848±5277 **

Fig. 3. Immune suppressor cells are induced after infusion of HbV (or empty liposomes). a, HbV- or EV-loaded bulk spleen cells (1×10^5) were mixed with control bulk spleen cells (1×10^5) and stimulated with Con A (0.3 μg/ml). T cell proliferation was clearly suppressed. **, $p < 0.01$. Data are representative of two independent experiments and are expressed as mean ± S.D. ($n = 3$). b, FITC-labeled empty liposomes (FITC-liposome) were injected into rats; spleens were excised the next day. Subsequently, single spleen cells were analyzed using FCM. Cells that phagocytized FITC-liposome were recognized clearly as FITC-positive cells. These cells (top square) were sorted using FCM. Their purity was >95%. c, control bulk splenocytes (2×10^5/well) were stimulated with Con A (0.3 μg/ml) in the presence of the sorted cells at the indicated ratios. Control bulk splenocytes was used as control cells. Splenic T cell proliferation was suppressed dose-dependently by FITC-positive cells (liposome-phagocytized cell). Data are representative of two independent experiments and are expressed as mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with control. *, $p < 0.05$ compared with control. d, T cell proliferation of FITC-negative cells (bottom square on b) (2×10^5/well) and FITC-EV-loaded bulk splenocytes was compared with that of control splenocytes. No inhibition was observed in FITC-negative cells. Data are representative of two independent experiments and are expressed as mean± S.D. ($n = 3$). **, $p < 0.01$ compared with control. *, $p < 0.05$ compared with control.

day 7. This pattern resembled that of immune suppression induced by the infusion of HbV (Fig. 1c). Consequently, the accumulation of liposomal particles in the spleen must be related somehow with this temporary immune suppression. Actually, splenocytes acquired the ability to inhibit the proliferation of control splenocytes after the injection of HbV (or

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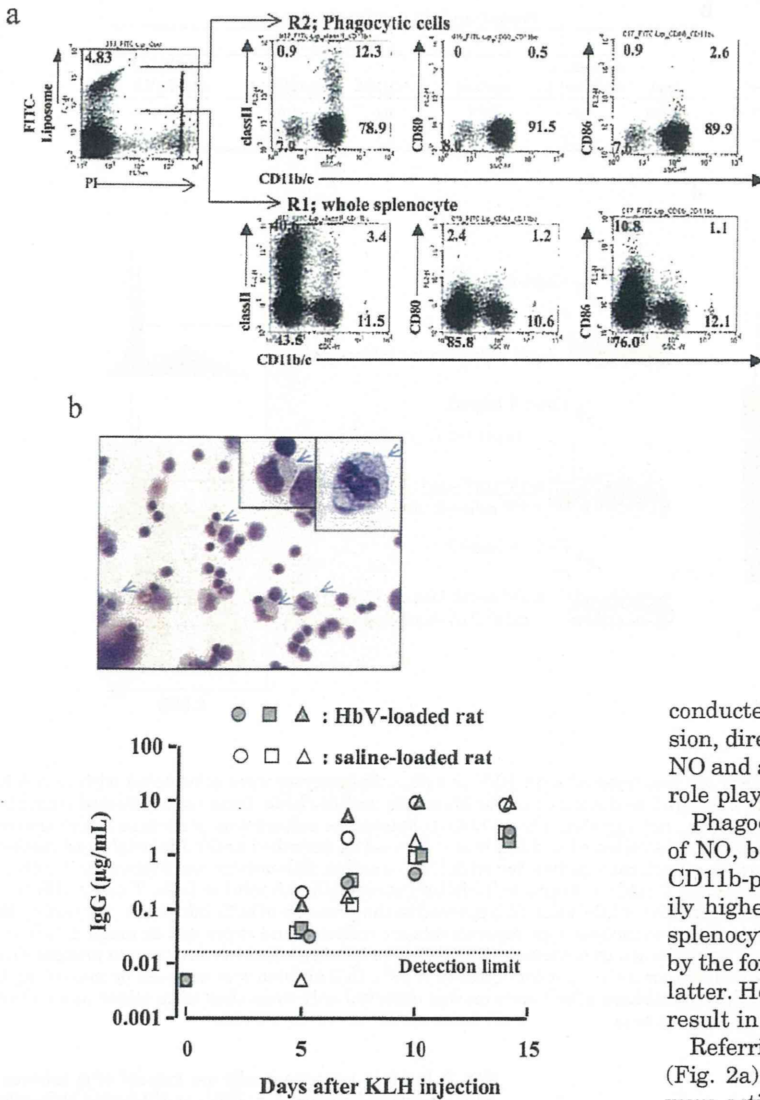

Fig. 4. Phenotypic and morphological analyses of FITC-liposome phagocytized cells. a, splenocytes derived from FITC-liposome-loaded rat spleen were analyzed. Top, FITC-positive cells were gated in R2. Bottom, whole splenocytes were gated in R1. Subsequently, the gated cells were analyzed for the indicated cell surface markers. More than 90% of FITC-positive cells were positive for CD11b/c, only partially positive for class II, and negative for CD80 and CD86, and HIS48. Data are representative of at least three independent experiments. b, HbV-loaded splenocytes were spun on a slide glass and stained with May-Grunwald-Giemsa dye. Images were visualized using a light microscope (BX50; Olympus) equipped with a 40×/0.75 or 100×/1.3 oil objective lens to give the original magnification at the time of photomicroscopy as ×400 and ×1000, respectively. Arrows indicate unique cells that appeared after the injection of empty vesicles. They seemed to be monocytic cells.

Fig. 5. Effect of HbV on primary antibody response. Primary antibody response to KLH was evaluated as described under *Materials and Methods*. The kinetics of the primary antibody response of three rats preloaded with HbV was shown to be comparable with that of three rats preloaded with saline.

EV) (Fig. 3a). Moreover, cells that trap liposomal vesicles (FITC-liposome) were shown to be responsible for immune suppression (Fig. 3,b and c). The result that cells sorted as FITC-negative cells acquired greater proliferative capacity might support this inference (Fig. 3d). Therefore, it was concluded that the cells responsible for the immune suppression were phagocytic cells. The expression of CD11b/c, which is a hallmark of macrophage/monocyte lineage, was consistent with the conclusion. Furthermore, their lack of class II molecule, CD80, and CD86 expression, coupled with their morphology (Fig. 4, a and c), indicated that they are immature monocytic cells. It is possible that they lost these molecules when phagocytizing liposomes. However, this might not be the case because, even in control splenocytes, most CD11b/c+ cells were negative for class II molecules, CD80, and CD86 (data not shown).

Based on the results obtained from a series of experiments conducted to elucidate the mechanism for immune suppression, direct cell-to-cell contact (Fig. 2c) is necessary, and both NO and arginase are involved (Fig. 2d), with a more principal role played by the former.

Phagocytosis of HbV might not be linked to the generation of NO, because the percentage of iNOS-positive cells among CD11b-positive cells (macrophage lineage) was not necessarily higher in HbV-loaded splenocytes than in saline-loaded splenocytes (data not shown). It is unclear why NO generated by the former tended to be higher than that generated by the latter. However, it is possible that phagocytosis of HbV might result in enhancing the generation of NO.

Referring to T cells, based on data about IL-2 secretion (Fig. 2a) and CD25 expression, HbV-loaded splenic T cells were activated in a normal way. However, they were unable to proliferate. It must be emphasized that these mechanisms and T cell status closely resemble those of T cell suppression caused by myeloid-derived suppressor cells (Mazzoni et al., 2002; Ostrand-Rosenberg and Sinha, 2009), although most liposome-phagocytizing cells did not express HIS 48 antigen, which is reportedly a marker of rat myeloid-derived suppressor cells (Dugast et al., 2008).

As described previously, the suppressive effect of HbV tended to be milder than that of EV (Fig. 1a). The Hb molecule might be responsible for the difference because the only difference between them was the presence of HbV. It is possible that NO is trapped by Hb molecules, engendering the decrease of its immunosuppressive effect. This trapping phenomenon might also explain why the suppressive effect of DPPC-liposome is more prominent than that of HbV (Fig. 6c).

Whether HbV infusion induced systemic immune suppression or not is the next concern of this research. The antibody response to KLH depends on T cells. Therefore, the primary antibody response to KLH was evaluated as a systemic immune response. The primary antibody response was unaffected by infusion of HbV, at least in our experimental conditions (Fig. 5). Therefore, it is less likely that massive

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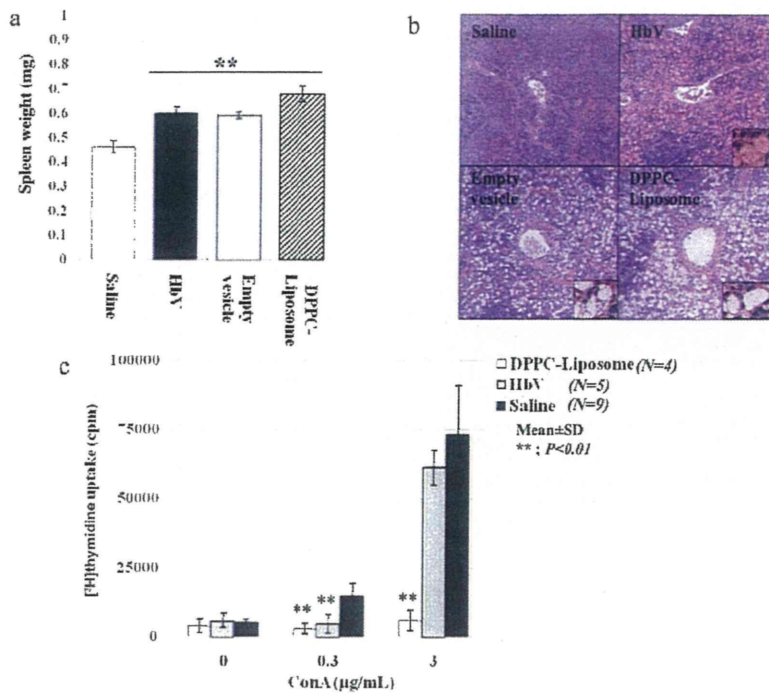

Fig. 6. Effect of DPPC-liposome on immune suppression. HbV, EV, DPPC-liposome, or saline was injected intravenously. The spleen was excised 18 h later. a, the spleen mass increased more after injection of HbV, EV, and DPPC-liposomes than after injection of saline alone. Data are expressed as mean ± S.D. (n = 3). **, $P < 0.01$. b, microscopic examination of the spleen injected with each vesicle. Unique cells with small particles in their enlarged cytoplasm appeared. The cellular cytoplasm of HbV-loaded spleen appeared to be reddish compared with that of other spleen, possibly because of Hb. Images were visualized using a light microscope (BX50; Olympus) equipped with a 20×/0.50 or 100×/1.3 oil objective lens to give the original magnification at the time of photomicroscopy as ×200 and ×1000. c, infusion of DPPC-liposome induced immune suppression. Data from several independent experiments were collected and expressed as mean ± S.D.

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infusion of HbV induces severe immunosuppressive effect on the host. The findings that T cell suppression was a transient phenomenon and that immune suppression in the lymph node cells was rather milder than that of splenocytes (Fig. 1b) support this. However, this finding must be emphasized: transient induction of immunosuppressive activity in HbV-phagocytizing cells is unique. Considering that infusion of HbV induces no strong adverse reaction, HbV (or liposomes) might be preferred as an immunosuppressive agent in certain clinical settings.

In conclusion, we demonstrated the existence of a subset of CD11b/c+, class II immature monocytes in rat spleen that can swiftly and transiently acquire strong T cell-suppressive potential via the phagocytosis of a considerable amount of HbV or DPPC-liposomes. Direct cell-to-cell contact and both iNOS and arginase are involved in that suppression. Immune suppression might be restricted to a local site such as the spleen, which has abundant phagocytic cells.

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Authorship Contributions

Participated in research design: Takahashi, Azuma, Sakai, Sou, and Fujihara.

Conducted experiments: Takahashi, Azuma, Wakita, and Abe.

Contributed new reagents or analytic tools: Sakai and Sou.

Performed data analysis: Takahashi, Azuma, Fujihara, Horinouchi, Nishimura, and Ikeda.

Wrote or contributed to the writing of the manuscript: Takahashi, Azuma, and Sakai.

Other: Horinouch and Kobayashi acquired funding for the research.

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Fluid Resuscitation with Hemoglobin Vesicles Prevents *Escherichia coli* Growth via Complement Activation in a Hemorrhagic Shock Rat Model

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ABSTRACT

Hemoglobin vesicles (HbVs) could serve as a substitute for red blood cells (RBCs) in resuscitation from massive hemorrhage. A massive transfusion of RBCs can increase the risk of infection, which is not caused by contaminating micro-organisms in the transfused RBCs but by a breakdown of the host defense system. We previously found that complement activity was increased after resuscitation with HbVs at a putative dose in a rat model of hemorrhagic shock. It is known that complement system plays a key role in host defense in the embryonic stage. Therefore, the objective of this study was to address whether the suppression of bacterial infections in hemorrhagic shock rats was a result of increased complement activity after massive HbV transfusion. For this purpose, *Escherichia coli* were incubated with plasma samples obtained from a rat model of hemorrhagic shock resuscitated by HbVs or RBCs, and bacterial growth was determined under ex vivo conditions. As a result, *E. coli* growth was found to be suppressed by increased complement activity, mediated by the production of IgM from spleen. However, this antibacterial activity disappeared when the *E. coli* were treated with complement-inactivated plasma obtained from splenoctomized rats. In addition, the resuscitation of HbVs from hemorrhagic shock increased the survival rate and viable bacterial counts in blood in cecum ligation and puncture rats, a sepsis model. In conclusion, the resuscitation of HbVs in the rat model of hemorrhagic shock suppresses bacterial growth via complement activation induced by IgM.

Introduction

Hemoglobin vesicles (HbVs), which were developed for use as an artificial oxygen carrier, have a cellular structure similar to that of red blood cells (RBCs), in that they are highly concentrated Hbs encapsulated in a phospholipid bilayer membrane with polyethylene glycol (Sakai et al., 2009b). HbV has been shown to possess a number of positive characteristics: the absence of viral contamination (Sakai et al., 1993), a long-term storage period of more than 2 years at room temperature (Sakai et al., 2000b; Sou et al., 2000), low toxicity (blood compatibility, no nephrotoxicity) (Sakai et al., 2000a), and good metabolic performance (Taguchi et al., 2009b, 2010). Moreover, the pharmacological effects of HbVs have been reported to be equivalent to that of RBCs, when evaluated in a model of hemorrhagic shock in rats (Sakai et al., 2004b, 2009a). In addition, the retention of HbVs in the circulation compares favorably with other artificial oxygen carriers (Taguchi et al., 2009b), and the half-life in humans is predicted to be sufficiently long to allow autologous blood to be recovered after a massive hemorrhage (Taguchi et al., 2009a). Based on these facts, it would be predicted that HbVs would be superior to a conventional blood transfusion and have considerable promise for use as a RBC alternative in patients with massive hemorrhages.

In modern medical care, there is now little doubt that the transfusion of RBCs is the gold standard for the treatment of patients with massive hemorrhages. However, such massive infusions are associated with numerous and significant com-

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ABBREVIATIONS: HbV, hemoglobin vesicle; RBC, red blood cell; wRBC, washed RBC; HbV_{4day}, 4 days after resuscitation by HbVs; HbV_{7day}, 7 days after resuscitation by HbVs; RBC_{4day}, 4 days after resuscitation by RBCs; RBC_{7day}, 7 days after resuscitation by RBCs; CH50, complement titer; ROS, reactive oxygen species; SD, Sprague-Dawley.

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plications, which include acute hemolytic transfusion reactions, bacterial sepsis, microchimerism, and transfusion-related acute lung injuries. Among these drawbacks, bacterial infections, which are not initially present as contaminants in transfusion solutions but invade afterward, remain a serious problem in the cases of injured and postoperative patients. In fact, it was reported that an increased risk of infection was as high as 15 to 40% in critically ill patients after massive transfusions (Hill et al., 2003; Rachoin et al., 2009). Because the increased incidence of bacterial infections causes increased morbidity and mortality, total hospital costs, and the total length of hospital stay, decreased incidences of bacterial infections in massive hemorrhagic patients would contribute, not only to a reduction in medical costs, but also to the enhancement of the quality of life of the affected patients.

One of the possible mechanisms for the increased rate of infection is the loss of immune system components/cells caused by hemorrhage. In addition, it is known that the increased rate of infection after massive hemorrhage is related to both the immunosuppression and breakdown of the gut barrier. The mechanism for the gut barrier breakdown is induced by ischemia reperfusion of small intestine. The precise mechanisms for immunosuppression remain uncertain, but increased bacterial infection rate is a known consequence of the breakdown of the host defense system (Sihler and Napolitano, 2010). The host defense system is comprised of two major effector systems that act immediately: 1) the complement system and the cellular system, which is composed of macrophages and neutrophils and 2) the adaptive immune system comprised of antigen-specific T and B lymphocytes. The complement represents the first line of defense for the host defense system and is the first contact with an infectious agent during the adaptive antigen-specific response. Therefore, the complement system is an important factor in the embryonic stage of development. In previous studies, we reported that IgM was induced by the resuscitation of HbVs at a putative dose, and the highest value was found at 4 days. It is well known that IgM is related to complement activation via the classic pathway. Therefore, it is possible that a massive transfusion of HbVs could result in the enhancement of the host defense system, as the result of an increase in complement activity, which would then suppress bacterial infections.

In this study, we hypothesized that the administration of HbVs under conditions of a massive hemorrhage would suppress the bacterial growth resulting from an increase in complement activity. For this purpose, we developed a rat model of hemorrhagic shock, and it was then resuscitated by HbVs or washed RBCs (wRBCs) at a putative dose of 1400 mg Hb/kg. At 4 and 7 days after resuscitation by HbVs (HbV_{4day}, HbV_{7day}) and wRBCs (RBC_{4day}, RBC_{7day}), we then evaluated the effects of complement titer (CH50) and plasma IgM on bacterial growth in ex vivo conditions. In addition, to confirm the effects of antibacterial activity under in vivo conditions, we created a double-hit model of hemorrhagic shock by cecum ligation and a puncture rat model, which is arguably a situation that closely replicates the nature and course of clinical sepsis in patients after trauma, and investigated the survival and viable bacterial counts in blood samples.

Materials and Methods

Preparation of HbV and wRBC Solution. HbVs were prepared under sterile conditions as reported previously (Sakai et al., 1997). The Hb solution was purified from outdated donated blood, which was provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM pyridoxal 5′-phosphate (Sigma-Aldrich, St. Louis, MO) as an allosteric effector to regulate the P_{50} to 25 to 28 Torr. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan) at a molar ratio of 5/5/1 and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-polyethylene glycol (NOF Corp., Tokyo, Japan) (0.3 mol%). The size of the HbVs was controlled at approximately 250 nm using an extrusion method. The HbVs were suspended in physiological salt solution at 10 g/dl [Hb], filter-sterilized (Dismic; Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with N_2 for storage.

To prepare wRBCs, blood samples from donated Sprague-Dawley (SD) rats (Kyudou Co., Kumamoto, Japan) were withdrawn and centrifuged at 1200*g* for 15 min to obtain an RBC concentrate. This sample was then washed three times to remove plasma components. The Hb concentration was determined by the cyanometHb method using a Hemoglobin B-Test-Wako kit (Wako Pure Chemical, Osaka, Japan). The wRBC samples were suspended in a physiological salt solution at 10 g/dl [Hb].

Before all of the experiments, HbVs or wRBCs were mixed with recombinant human serum albumin (Nipro Corp., Osaka, Japan) to adjust the albumin concentration of the suspension medium to 5 g/dl. Under these conditions, the colloid osmotic pressure of the suspension is maintained constant at approximately 20 mm Hg (Sakai et al., 2004b).

Preparation of Hemorrhagic Shock Model Rats. All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. SD rats were maintained in a temperature-controlled room with a 12-h dark/light cycle and ad libitum access to food and water. Hemorrhagic shock model rats were prepared as described previously (Taguchi et al., 2009a). In brief, SD rats (180–210 g) were anesthetized with pentobarbital. Polyethylene catheters (PE 50 tubing, o.d. equal to 0.965 mm, and i.d. equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were then introduced into the left femoral artery for infusion and blood withdrawal. Hemorrhagic shock was induced by removing 40% of the total blood volume (22.4 ml/kg). Systemic blood volume was estimated to be 56 ml/kg (Sakai et al., 2004b). After removing the blood, the hemorrhagic shock rats were resuscitated by an infusion of either isovolemic HbVs or wRBCs (1400 mg Hb/kg, 22.4 ml/kg). After resuscitation, the polyethylene catheter was removed, the femoral artery was ligated, and the skin was sutured with a stitch. The animals were housed in a temperature-controlled room with a 12-h dark/light cycle and ad libitum access to food and water. The arterial blood oxygen tension (PaO_2) and pH were evaluated before hemorrhage with the left femoral artery and 4 and 7 days after resuscitation with the right femoral artery. The i-STAT system (Abbott Laboratories (Abbott Park, IL) was used for analyses of PaO_2 and pH.

Sample Collection. At 4 or 7 days after resuscitation from hemorrhagic shock by HbV and wRBC solution, blood samples were collected from tail vein, and plasma samples were obtained by centrifugation (3000*g*, 5 min). Antibacterial activity was immediately measured, and the remaining samples were stored at −80°C until use in measurements of CH50 and plasma IgG and IgM.

The Evaluation of Antibacterial Activity. In vitro antibacterial activities were determined by previously reported methods with minor modification (Ishima et al., 2007). M9 medium, pH 7.4, was used during the incubation of bacteria with serum. In a typical run, *Escherichia coli* ATCC strains were cultured overnight in M9 me-

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dium. A 50-μl bacterial suspension in M9 medium (OD_{630}; 0.05 ± 0.01) was placed in a 96-well plate, and 50-μl aliquots of plasma samples were added. After 6 h of incubation at 37°C, the number of bacteria exposed to various plasma samples were determined by measuring the absorbance 630 nm using a microplate reader (model 450; Bio-Rad Laboratories, Hercules, CA).

Surgery for Splenectomy. For splenectomy, the rats were anesthetized using pentobarbital and, after shaving, an incision was made in the skin at the left flank. The peritoneal membrane was opened, and the entire spleen was removed intact after ligating the splenic vein and artery at the hilum. The peritoneal membrane and skin were separately sutured (Ishida et al., 2006). One week after surgery for splenectomy, the rats were used in experiments.

Measurement of Plasma IgG, IgM, and CH50. The plasma samples obtained 4 and 7 days after resuscitation by the administration of HbV and wRBC solutions were stored at −80°C before analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan). CH50 was measured in hemolytic assays as described previously (Mayer, 1961).

Measurement of Phagocyte Activity. Phagocyte activity was determined by the carbon clearance method, as described previously (Taguchi et al., 2011b). Hemorrhagic shock was induced in the rats, which were then resuscitated with HbVs and wRBCs. In a typical experiment, rats were anesthetized with pentobarbital. Polyethylene catheters (PE 50 tubing) containing saline and heparin were then introduced into the left femoral vein for the infusion of a carbon particle solution and blood collection. The carbon particle solution (Fount India Ink; Pelikan Co., Hannover, Germany) was infused at 10 ml/kg within 1 min. At 4, 10, 20, 30, 45, and 60 min later, approximately 100 μl of blood was withdrawn, and exactly a 50-μl aliquot was diluted with 5 ml of a 0.1% sodium bicarbonate solution. The absorption was measured at 675 nm by means of a spectrophotometer (U-2900; Hitachi, Tokyo, Japan). The phagocyte index (K) was calculated using the equation: $K = 1/(t_2 - t_1) \times \ln(C_1/C_2)$, where C_1 and C_2 are the concentrations (absorbance) at time t_1 and t_2 (min), respectively.

Preparation of Cecum Ligation and Puncture Model and the Measurement of Viable Bacterial Counts in Blood. Bacterial infection was induced by cecum ligation and puncture, following a previously reported method with minor modification (Hubbard et al., 2005). Hemorrhagic shock was induced in the rats, which were then resuscitated with HbVs and wRBCs. Four or 7 days after resuscitation with HbVs and wRBCs, the cecum was ligated without preventing passage of gastric contents. Ten punctures in cecum ligation were then performed using a 21-gauge needle. Survival rate was determined daily for 7 days after puncture. The blood samples from surviving rats (0.3 ml) were collected from tail vein 24 h after the puncture. After collecting blood samples, the aliquot blood (0.1 ml) was immediately placed on Luria-Bertani agar plates (9.9 ml) and the plates were incubated at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as colony-forming units/ml.

Data Analysis. Data are shown as the means ± S.D. for the indicated number of animals. Significant differences among each group were determined using the two-tail unpaired Student's t test. The Spearman test was used for correlation analyses. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

Results

Survival Rate and Blood Gasses Analysis. All hemorrhagic shock rats resuscitated by an infusion of either isovolemic HbVs or wRBCs (1400 mg Hb/kg, 22.4 ml/kg) survived during experiments and were well tolerated with no apparent change in behavior. The PaO_2 before induction of hemorrhagic shock by bleeding was 88.8 ± 5.7 Torr and increased significantly to 129.3 ± 17.4 Torr after induction of hemorrhagic shock. After resuscitation with HbVs or wRBCs, PaO_2 recovered similar to PaO_2 measured at baseline (76.3 ± 7.8 and 93.5 ± 16.5 Torr for 4 and 7 days after HbV resuscitation, respectively; 86.8 ± 14.8 and 87.8 ± 11.1 Torr for 4 and 7 days after wRBC resuscitation, respectively). In addition, pH before and after hemorrhage was 7.33 ± 0.07 and 7.28 ± 0.08, respectively, and it recovered by resuscitation with HbVs (7.37 ± 0.06 and 7.43 ± 0.05 for 4 and 7 days after resuscitation, respectively) or wRBCs (7.41 ± 0.03 and 7.44 ± 0.02 for 4 and 7 days after resuscitation, respectively). The data showed similar tendency as that reported previously by Sakai et al. (2009a).

The Evaluation of Antibacterial Activity. To evaluate antibacterial activity, the collected plasma samples were incubated with *E. coli* (ATCC strain) for 6 h. As shown in Fig. 1A, the only plasma sample in HbV_{4day} showed a dramatic level of antibacterial activity, whereas other plasma samples obtained from the hemorrhagic shock rats did not show any activity. Because it is possible that HbV directly suppresses *E. coli* growth, HbV alone was incubated with *E. coli* (0, 0.049, 0.098, 0.195, 0.391, and 0.781 mg Hb/ml). As a result, HbVs did not show any antibacterial activity during 6 h (data not shown). This result clearly excluded the possibility of HbV itself directly contributing to the antibacterial activity observed in plasma samples from the hemorrhagic shock rats that had been resuscitated by HbV. Consequently, other factors in plasma induced by resuscitation with HbV seem to be indirectly involved in this unique phenomenon.

The Relationship between CH50 and Antibacterial Activity. In a previous study, we reported that CH50 in plasma was decreased after resuscitation with HbV in the rat model of hemorrhagic shock (Taguchi et al., 2011a). Because it is well known that complement represents the first line of the host defense system and possesses bacteriolytic effects, we examined the relationship between the antibacterial activity described above and CH50 in plasma.

As shown in the previous results, the CH50 for HbV_{4day} was significantly smaller than that in normal rats, whereas the other samples remained unchanged ($p < 0.01$; Fig. 1B). As shown in Fig. 1C, CH50 was well correlated with antibacterial activity ($r = 0.47$, $p = 0.013$). These results strongly suggest that CH50 is associated with the observed antibacterial activity.

The Relationship between Plasma IgM and Antibacterial Activity. Because Ishida and Kiwada (2008) demonstrated that the IgM produced from spleen is related to the reduction of CH50 after the administration of liposomes, it is possible that the change in plasma IgM levels after HbV administration might affect antibacterial activity via the reduction of CH50.

As shown in Fig. 2A, the plasma IgM level in the HbV_{4day} was significantly larger than that in normal rats, but the other plasma samples were not significantly changed ($p < 0.01$; Fig. 3A). Similar to CH50 (Fig. 1B), plasma IgM levels were well correlated with the observed antibacterial activity ($r = 0.64$, $p = 0.0006$; Fig. 2B). To further demonstrate this relationship, we prepared splenectomized rats to suppress the production of IgM by the administration of HbV, and the same experiments were then performed. The level of plasma IgM and CH50 were maintained at the normal rat level by

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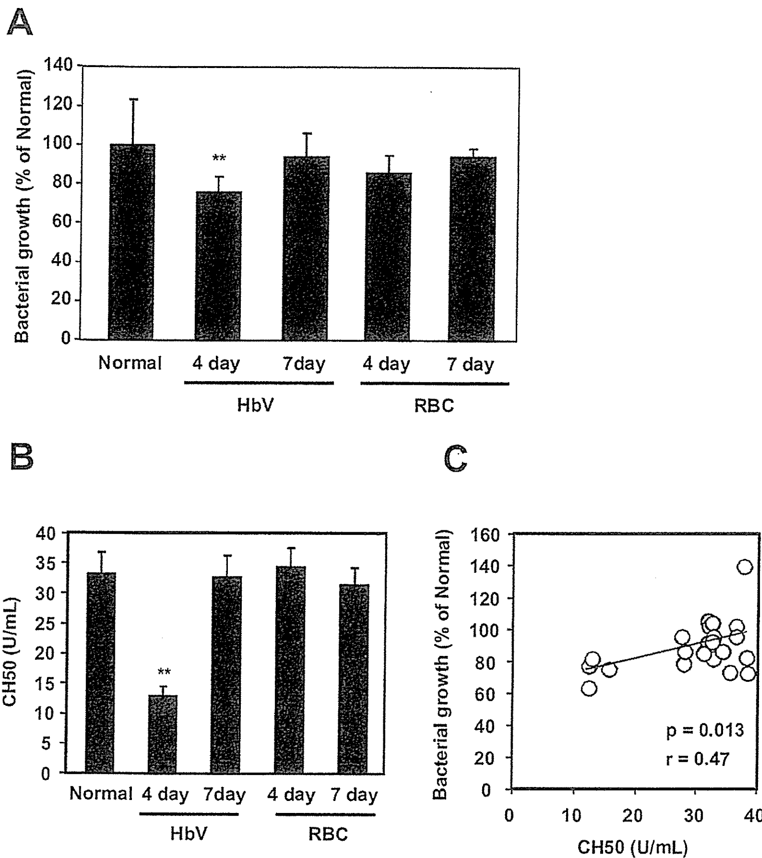

Fig. 1. A, bacterial (*E. coli* ATCC strains) growth rate after resuscitation with HbVs or wRBCs in a rat model of hemorrhagic shock. Blood was collected from the tail vein, and plasma was obtained. *E. coli* ATCC strains and each plasma sample were mixed (6 h, 37°C). Bacteria growth was determined by measuring the absorbance at 630 nm. Each bar represents the mean ± S.E.M. (n = 4). **, $p <$ 0.01 versus normal rats. B, the measurement of CH50in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation. Plasma samples were obtained at 4 and 7 days after resuscitation with HbV and wRBC. Each bar represents the mean ± S.D. (n = 4). **, $p < 0.01$ versus normal rats. C, relationship between CH50 and bacterial growth. The linear regression was calculated using the least-squares method ($y = 0.892x$ 64.9, $r = 0.47, p = 0.013$).

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removing the spleen; even in 4 days after resuscitation with HbV (Fig. 3, A and B), no significant antibacterial activity was detected in any of the splenectomized rats (Fig. 3C). Consequently, no significant correlation was found between either plasma IgM and antibacterial activity or CH50 and antibacterial activity (r = 0.18 and 0.03, for plasma IgM and complement activity, respectively) (data not shown).

Evaluation of Antibacterial Activity in the Two-Hit Model of Hemorrhagic Shock and Sepsis Rat Model Induced by Cecum Ligation and Puncture. To examine whether the antibacterial activity caused by resuscitation with HbV were observed under conditions of bacterial infection in vivo, we prepared a double model of hemorrhagic shock and cecum ligation and puncture, as a model for sepsis, arguably one that closely replicates the nature and course of clinical sepsis in patients after trauma (Hubbard et al., 2005).

Figure 4A shows the survival rates after the induction of cecum ligation and puncture. Ninety percent of the normal rats died within 36 h after the induction of cecum ligation and puncture. However, the survival times for HbV_{7day}, RBC_{4day}, and RBC_{7day} rats were prolonged, but 90% of these died within 120 h. It is noteworthy that a significant number of animals at HbV_{4day} survived compared with normal rats, and 40% of the rats in this group survived until 168 h after the induction of cecum ligation and puncture. We also measured the amount of bacteria in blood at 24 h after the induction of cecum ligation and puncture. As shown in Fig. 4B, the amount of bacteria in blood decreased in the HbV_{4day} and HbV_{7day} rats compared with the normal and RBC resuscitation groups. To investigate whether the amount of bacteria in blood had an influence on survival rate, we compared the numbers of bacteria in blood between the rats that survived for more than 72 h and the rats that died within 72 h. As shown in Fig. 4C, the amount of bacteria in the blood of the rats that survived more than 72 h was less than that in the rats that died within 72 h. These results indicate that the elevated complement activity after HbV administration contributed to the removal of bacteria, even under conditions of severe sepsis, and the clearance of bacteria at an earlier time was an important factor in the improved survival rates.

Phagocyte Activity. Phagocyte activity, especially in Kupffer cells, is strongly related to the removal of bacteria in vivo. Thus, to investigate the effect of resuscitation by HbV and wRBC solutions on phagocyte activity in Kupffer cells, we estimated the in vivo carbon clearance, an indicator of phagocyte activity in Kupffer cells [Kupffer cells engulfed more than 90% of the injected carbon particles (Zweifach and Benacerraf, 1958)].

As shown in Table 1, phagocyte activity in HbV_{4day} and HbV_{7day} rats was approximately 1.5 and 1.9 times higher than that in normal rats. In contrast, phagocyte activity for both RBC_{4day} and RBC_{7day} rats was comparable with that in normal rats. These data indicate that phagocyte activity was increased after resuscitation with HbV in the rat model of hemorrhagic shock, and the enhanced phagocyte activity played a role in the clearance of bacteria in vivo.

Discussion

The "two-hit" theory proposes that a host primed by an initial stress such as a massive hemorrhage is likely to show

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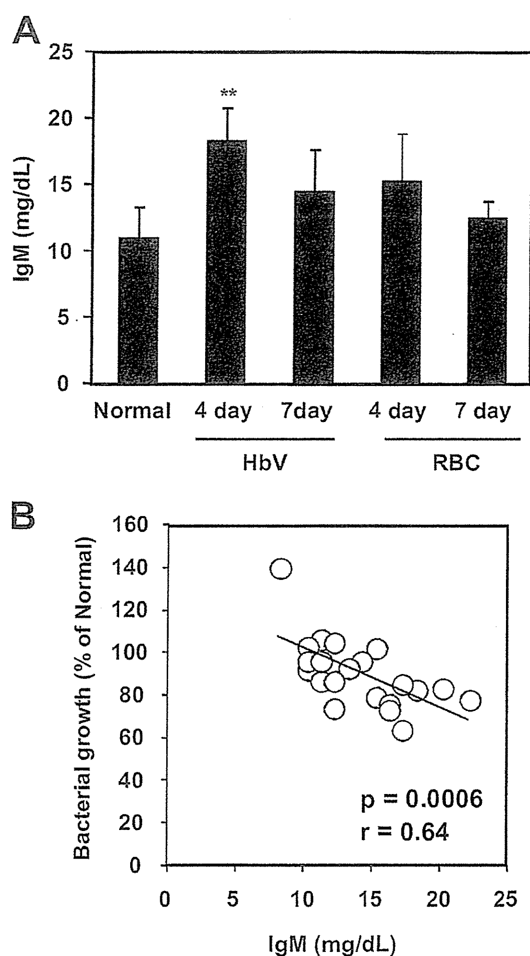

Fig. 2. A, the measurement of plasma IgM concentrations in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation. Plasma samples were obtained at 4 and 7 days after resuscitation with HbV and wRBC solution. The plasma samples were then ultracentrifuged to remove intact HbV (50,000*g*, 30 min). Each bar represents the mean ± S.D. ($n = 4$). **, $p < 0.01$ versus normal rats. B, relationship between plasma IgM concentration and bacterial growth. The linear regression was calculated using the least-squares method ($y = -2.77x \quad 129.8$, $r = 0.64$, $p = 0.0006$).

an abnormal response to a second stress such as an infection (Price et al., 1999). It has been shown that bacterial translocation from the gut to mesenteric lymph nodes induced by hemorrhagic shock, in combination with the immunosuppressive effects of hemorrhage, can amplify the second hit (Turnbull et al., 1995). In fact, Rachoin et al. (2009) reported that the approximately 10% of transfused patients developed nosocomial infections, and the number of patients infected with *E. coli* was similar to that of methicillin-resistant *Staphylococcus areus*. *E. coli* is classed as a gram-negative bacteria, in which the outer membrane contains a lipopolysaccharide as endotoxin, which causes endotoxin shock (sepsis) and multiple organ failure (Yang and Lee, 2008). Therefore, in terms of decreasing mortality and morbidity among transfused patients with massive hemorrhage, the removal of Gram-negative bacteria, especially *E. coli*, at an early phase is an important issue.

The findings reported herein showed that plasma of rats resuscitated with HbVs from hemorrhagic shock showed a suppressed *E. coli* growth correlated to reduction of CH50 (Fig. 1). Four possible mechanisms for the reduction of CH50 after HbV resuscitation can be proposed: 1) The liver injury decreases the production of complement; however, it was reported that HbVs did not directly injure the liver and other organ. Therefore, it would affect the production of complement. 2) The direct effect of HbV, which makes contact with complement, subsequently induces the complement activation, because it was previously reported that liposomes containing cholesterol induce complement activation (Alving et al., 1977; Cunningham et al., 1979). However, in the case of HbV, the level of CH50 was equivalent to that observed for saline treatment in both in vitro studies using human serum (Abe et al., 2006) and in vivo using healthy rats (Sou and Tsuchida, 2008). In addition, *E. coli* incubated with HbVs did not show antibacterial activity (data not shown). Therefore, it does not seem the HbV itself directly affects the reduction of CH50. 3) Plasma IgG, which activates complement by binding to an antigen, recognizes microbial polysaccharides, and by the lipid A component of lipopolysaccharide. However, plasma IgG was hardly induced by HbV administration, and no correlation with the antibacterial activity was found ($r = 0.09$, data not shown). 4) Plasma IgM, which activates complement via the same pathway as plasma IgG, as mentioned above. In this study, the plasma IgM produced in spleen was well correlated with *E. coli* growth (Fig. 2). Furthermore, it was observed that both CH50 and antibacterial activity disappeared by suppression of IgM production using splenoctomized rats (Fig. 3). Based on these results, it seems that complement is activated by binding IgM to *E. coli*, and this complement activity would result in suppressed *E. coli* growth.

The complement system contributes to host defense against infections by two different mechanisms: C3-mediated lysis, which leads to the formation of a membrane attack complex (Frank et al., 1987), and opsonic activity, which leads to phagocytosis by macrophages and Kupffer cells (Esser, 1994). Therefore, C3 is a key factor for antibacterial activation. In fact, a clinical C3 deficiency involves the loss of major complement opsonin and failure to activate the membrane attack complex pathway (Walport, 2001). It was previously reported that when liposomes are repeatedly injected into the same animal IgM is induced by the first injected liposomes and the binding of IgM to the second injected liposomes occurs, followed by C3 activation by IgM (Ishida and Kiwada, 2008; Taguchi et al., 2009c). Therefore, C3 could also be activated by the binding of IgM to *E. coli* in the present study.

In addition, we used a double-hit model of hemorrhagic shock and sepsis, which closely replicates the nature and course of clinical sepsis in patients after trauma (Hubbard et al., 2005). Under these conditions, only 10% of the normal and RBC resuscitation rats survived 168 h after the induction of cecum ligation and puncture, whereas 40% of the HbV_{4day} animals survived (Fig. 4A). Previously, Turnbull et al. (2004) reported that antimicrobial therapy with the broad-spectrum antibiotic imipenem at a dose of 25 mg/kg resulted in a significant improvement in survival rate (46%) compared with nontreatment (26%) in cecum ligation and puncture model mice. Furthermore, a imipenem-cilastatin treatment, administered 500 μg intraperitoneally every 12 h, prolonged the survival time in cecum ligation and puncture model mice, but did not prevent mortality (100% mortality;

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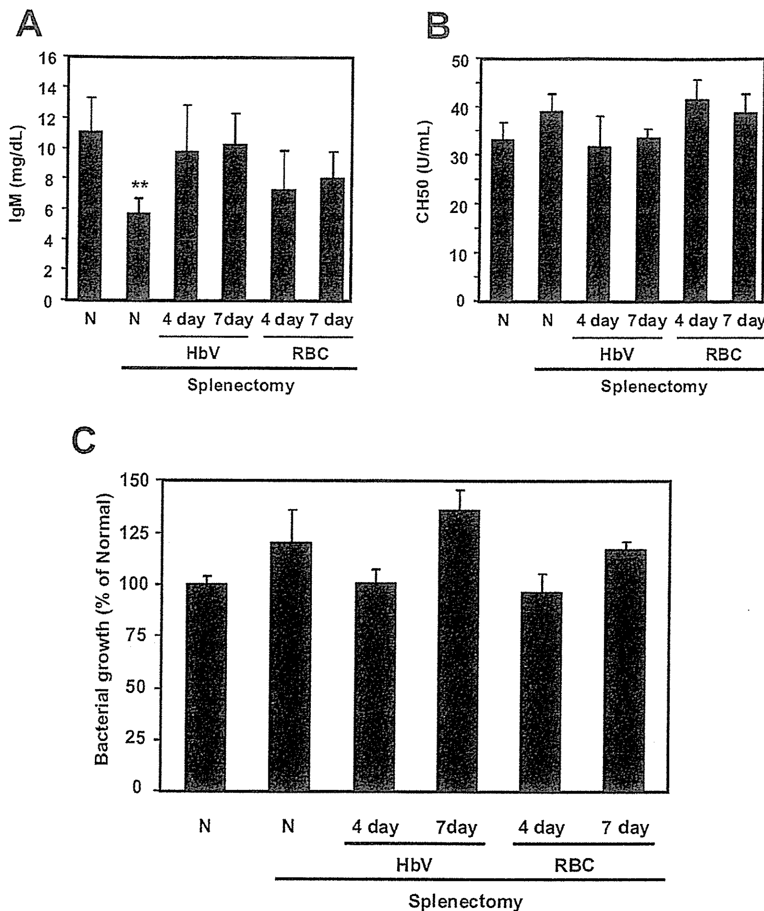

Fig. 3. A and B, the measurement of plasma IgM concentration (A) and CH50 (B) in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation in splenectomy rats. Plasma samples from splenectomy rats were obtained 4 and 7 days after resuscitation with HbV and wRBC solutions. The plasma samples were then ultracentrifuged to remove intact HbV (50,000*g*, 30 min). Each bar represents the mean ± S.D. ($n = 4$). C, the antibacterial (*E. coli* ATCC strains) activity of plasma obtained from splenectomy rats. *E. coli* ATCC strains and each plasma sample obtained from splenoctomized rats were mixed (6 h, 37°C). Bacteria growth was determined by measuring the absorbance at 630 nm. There was no significant difference among groups. Each bar represents the mean ± S.E.M. ($n = 4$).

84 and 120 h, for cecum ligation and puncture alone and imipenem-cilastatin treatment groups, respectively) (Doerschug et al., 2004). Therefore, it might be expected that complement activation by IgM after HbV resuscitation would achieve a full therapeutic effect for antibacterial activity, similar to antibiotic therapy.

In addition to the enhancement in survival rate, the amount of bacteria in blood of the HbV resuscitation groups was less than those of the other groups (Fig. 4B). In ex vivo conditions, although the HbV_{7day} group did not show antibacterial activity, the amount of bacteria in blood was suppressed. Because the mononuclear phagocyte system, especially Kupffer cells, plays a major role in the clearance of microorganisms (Bilzer et al., 2006), the suppression of bacterial count in the HbV_{7day} group seems to be the result of increased phagocyte activity. Actually, phagocyte activity in the HbV_{7day} rats was approximately 1.9 times higher than that in normal rats (Table 1).

It might be a concern that the elevated complement activity induced by IgM after HbV resuscitation damaged some host organisms. However, when the plasma samples were added to human umbilical vein endothelial cells, which are commonly used as a model of vascular endothelial cells, to examine cellular injury using WST-8, no significant difference in human umbilical vein endothelial cell viability was found among the groups at 6 or 24 h (data not shown). Therefore, it is unlikely that the observed complement activation damages host organisms. In fact, it was reported that histological changes and an enhancement in organ injury markers were not observed in the hemorrhagic shock rat model after resuscitation by HbV (Sakai et al., 2009a). Moreover, complement activation is a subject of great concern as a pseudoallergic reaction (Laverman et al., 2001). In this study, no allergic-like reactions, such as anaphylactic reactions, were observed, and previous reports demonstrated that healthy rats, which were repeatedly injected a massive dose of HbV (10 ml/kg/day for 14 days), all survived and showed no toxicity (Sakai et al., 2004a). Therefore, an enhancement in complement activity after HbV injection would not be expected to induce allergic reactions or have an effect on host organisms. However, it is possible that the HbV administration might produce detrimental outcome in conditions in which complement activation increases the risk of survival, because HbV enhanced the complement activation. In the case of using HbV clinically, resuscitation should be the first priority as in hemorrhagic shock that decreased the survival rate. Having said that, the effect of complement activation via HbV administration in conditions in which complement activation increases the risk of survival deserves further investigation.

Although the complement activation induced by IgM after HbV administration showed antibacterial activity, our model has several limitations with respect to providing a complete explanation for antibacterial activity. It is well known that reactive oxygen species (ROS) and cytokines, such as interleukin-6, tumor necrosis factor-α, and C-reactive protein, are

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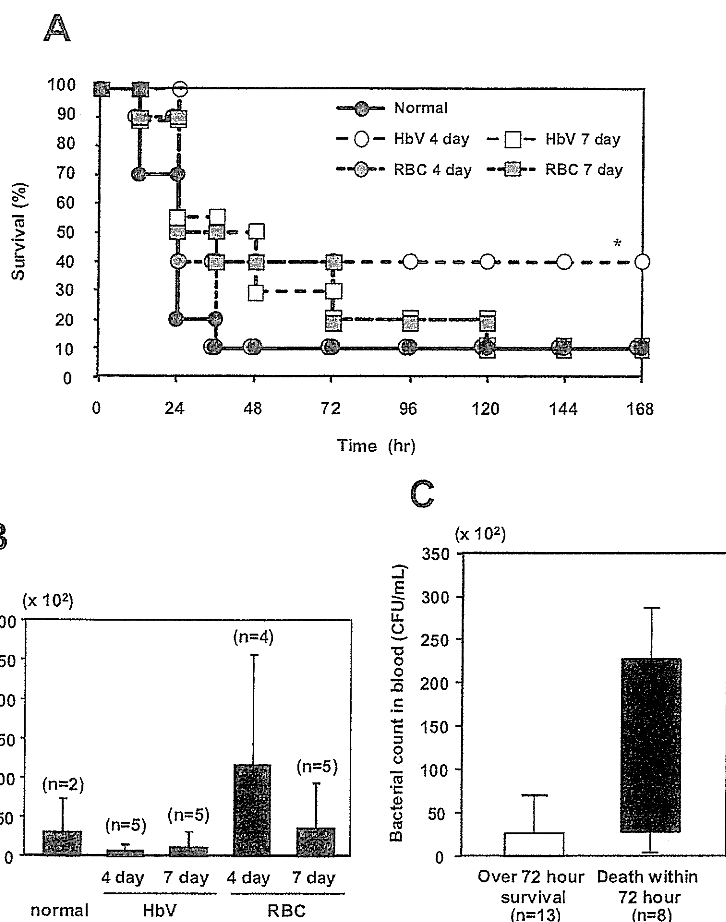

Fig. 4. A, survival rate of the two-hit model of hemorrhagic shock and sepsis. Sepsis was induced by cecum ligation and puncture. The cecum was ligated, and then 10 punctures in the cecum ligation were performed using a 21-gauge needle. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test. *, $p < 0.05$ versus normal rats ($n = 10$). B, amount of bacteria in blood at 24 h after cecum ligation and puncture. C, the comparison of the amount of bacteria in blood at 24 h after cecum ligation and puncture between rats that survived over 72 h and rats that died within 72 h after the induction of sepsis. Blood samples from survival rats (0.3 ml) were collected from the tail vein 24 h after puncture. After collecting the blood samples, aliquots (0.1 ml) were immediately placed on Luria-Bertani agar plates (9.9 ml) followed by incubation at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as colony-forming units (CFU)/ml. Each bar represents the mean ± S.D.

TABLE 1
Phagocyte activity in hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation

Carbon clearance was estimated, and K was calculated from the clearance of carbon particles. Results represent the mean ± S.D. ($n = 5$).

	Percentage of Normal	
	4 Days	7 Days
HbV	163 ± 14*	189 ± 38**
wRBC	83 ± 19	96 ± 19

* $P < 0.05$, ** $P < 0.01$ vs. normal rats.

also related to antibacteria action. In this study, the effect of ROS was estimated by determining the oxidative albumin ratio, which serves as a nonspecific marker of ROS (Shimoishi et al., 2007). As a result, we found that the relationship between ROS and antibacterial activity was significantly correlated ($r = 0.42, p < 0.05$; data not shown). Therefore, it is possible that ROS and cytokines are also involved in the antibacteria action observed here. However, further study will be needed to clarify the contribution of these factors. Moreover, in clinics, patients frequently become infected with various species of bacteria including Gram-negative and -positive bacteria, but we used only Gram-negative bacteria, *E. coli*. In general, Gram-positive bacteria are less sensitive to lysis by complement than Gram-negative bacteria (Mold, 1999). However, because Gram-positive bacteria affected the opsonization by complement, antibacterial activity of Gram-positive bacteria under in vivo conditions would not be unexpected. Further study will be needed to demonstrate this fact.

Based on the present findings, resuscitation with HbV in a rat model of hemorrhagic shock suppressed the growth of *E. coli* via complement activation induced by IgM, compared with wRBC resuscitation. However, this possible therapeutic efficacy was limited to 4 days after resuscitation with HbV. Fortunately, in clinical settings, the timing of infections from the first transfusion is typically approximately 4 to 5 days, which is consistent with the present results (Rachoin et al., 2009). Given the above findings and ongoing progress in antibacterial therapy, HbV resuscitation may contribute to a reduction of infections in patients with a massive hemorrhage, especially in cases of inadequately resourced health service areas.

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Authorship Contributions

Participated in research design: Taguchi, Watanabe, Maruyama, and Otagiri.

Conducted experiments: Taguchi and Ogaki.

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

Performed data analysis: Taguchi and Kadowaki.

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

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Intravenous infusion of Hb-vesicles (artificial oxygen carriers) after repetitive blood exchange with a series of plasma expanders (water-soluble biopolymers) in a rat model†

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Hemoglobin-vesicles (HbV) are artificial oxygen carriers developed for use as a transfusion alternative. The extremely high concentration of the HbV suspension (solutes, *ca.* 16 g/dl; volume fraction, *ca.* 40 vol%) provides a sufficient oxygen carrying capacity to maintain oxygen metabolism. A suspension of HbV has no colloid osmotic pressure (COP). Consequently, a combination of a plasma expander is necessary for a massive dose of HbV. Clinically available plasma expanders include hydroxyethyl starch (HES), modified gelatin (MFG), or recombinant human serum albumin (rHSA). Our previous studies confirmed that these water-soluble biopolymers interact with HbV to induce flocculation of HbV reversibly by depletion interaction, especially with MFG and high molecular weight HES. It remains unknown whether such flocculate formation in blood might affect animal's hemodynamics. Using a rat model, we tested infusion of a series of plasma expander to maintain the blood volume (level of blood exchange led to 60%) at repeated hemorrhages and the subsequent infusion of HbV (20 ml/kg, 36% of blood volume). All rats survived for 4 hr after the infusion of HbV; hemodynamic and respiratory functions were preserved, indicating that the flocculation does not induce capillary embolism. Blood exchange with rHSA and subsequent infusion of HbV showed more stable systemic parameters because of the longer retention of rHSA in blood than other plasma substitutes, indicating that rHSA is suitable for combination with HbV in this experimental model. **Copyright © 2011 John Wiley & Sons, Ltd.**

Keywords: blood substitutes; artificial red cells; biomacromolecules; hemoglobin; liposome

INTRODUCTION

Hemoglobin-vesicles (HbV) are artificial oxygen carriers that encapsulate a concentrated Hb solution in phospholipid vesicles (280 nm particle diameter). HbV has been developed by the Oxygen Infusion Project of Waseda University, directed by Prof. Eishun Tsuchida since 1990s from the view point of molecular assembly and macromolecular metal complexes.[1–3] The oxygen carrying capacity and safety of HbV as a transfusion alternative have been evaluated energetically in animal tests aimed at clinical applications. In contrast to conventional liposomal products, the concentration of the recent HbV suspension is extremely high (Hb, 10 g/dl). One infusion as a transfusion alternative causes the substitution of a large volume of blood: about 40% of the circulating blood volume.[4] Accordingly, it is important to evaluate its safety, not only in terms of the

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