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Alteration in the Pharmacokinetics of Hemoglobin-Vesicles in a Rat Model of Chronic Liver Cirrhosis Is Associated with Kupffer Cell Phagocyte Activity

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ABSTRACT: The hemoglobin-vesicle (HbV) is a cellular, hemoglobin-based oxygen carrier. Our previous pharmacokinetic studies demonstrated that the liver is strongly associated with the metabolism and excretion of HbV. The aim of this study was to evaluate the pharmacokinetics of HbV in a chronic cirrhosis rat (CCR) model induced by carbon tetrachloride (CCl_4) and explore whether liver functional parameters and Kupffer cell (KC) phagocyte activity are related to the pharmacokinetics of HbV. The CCRs were induced three times weekly by intraperitoneal administration of CCl_4 for 8 weeks and categorized as Child–Pugh grade B. To analyze the pharmacokinetics, the CCRs were given a single intravenous injection of ^{3}H-HbV (1400 mg of Hb/kg). The total clearance and hepatic distribution of HbV were negatively correlated with plasma aspartate aminotransferase (AST) levels ($p = 0.007$). In addition, the phagocyte index was negatively correlated with plasma AST levels ($p = 0.047$). The excretion of lipid components in feces was also negatively correlated with plasma AST levels ($p = 0.049$). In conclusion, alteration in the pharmacokinetics of HbV in CCRs can be attributed to a decrease in KC phagocyte activity and the extent of damage to parenchymal cells. This represents the first demonstration of the pharmacokinetics of a liposome preparation in chronic liver impairment. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:775–783, 2011

Keywords: pharmacokinetics; hepatocyte; biliary excretion; metabolism; liposomes

INTRODUCTION

The hemoglobin-vesicle (HbV) is an artificial oxygen carrier in which a concentrated hemoglobin solution is encapsulated in a liposome, the surface of which is modified with polyethylene glycol (PEG). HbV is potentially promising for use in clinical settings because its oxygen transport characteristics are comparable with red blood cells.[1,2] Moreover, HbVs possess a number of positive characteristics, including the absence of viral contamination,[3,4] capability of long-term storage for periods of more than 2 years at room temperature,[5–7] and low toxicity (good blood compatibility and low bioaccumulation potential),[8,9] and can control the release of oxygen in proportion to the amount of allosteric effector present.[10] In a previous study, we demonstrated that HbV and its components can be safely used in normal and hemorrhagic shock model rats from the viewpoint of pharmacokinetics.[9,11,12] These results were encouraging because pharmacokinetics

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data in animals can frequently be extrapolated to humans.

In clinical use, the possibility exists that HbV could be transfused to patients with chronic liver cirrhosis because it would be involved in upper gastrointestinal bleeding following the rupture of the portosystemic collaterals, the so-called variceal bleeding. We previously reported that the liver is a key organ in predicting the safety and efficacy of HbV in humans because HbV is mainly degraded by Kupffer cells (KCs) as well as other liposome preparations, and the lipid components of HbV, especially cholesterol, are excreted to feces via biliary excretion.[9,13] This indicates that HbV can be classified as a hepatically cleared and excreted drug. Because hepatic impairment affects the pharmacokinetics of drugs, including metabolism and excretion,[14] it appears likely that liver impairment would also affect the pharmacokinetics of HbV. Unfortunately, to our knowledge, no reports of pharmacokinetic studies of drugs that are metabolized by KCs (e.g., liposome preparations), under conditions of hepatic impairment, are available. Therefore, such an investigation would be desirable, not only to demonstrate the efficacy and safety of HbV but also to obtain other liposome preparations under conditions of liver failure, because it would enable predictions of the pharmacokinetics in patients with liver failure to be made, thus avoiding adverse drug reactions.

The aim of this study was therefore to assess the effect of hepatic impairment on the pharmacokinetics of HbV and explore the parameters of liver function that are related to the pharmacokinetics. Moreover, we also investigated the issue of whether the function of KCs affects the pharmacokinetics of HbV and whether they are related to liver function under *in vivo* conditions. Such relationships may be useful for determining the optimal dosage regimen for HbV in cases of liver failure. To achieve this, we produced a chronic cirrhosis rat (CCR) model, with fibrosis induced by the administration of carbon tetrachloride (CCl_4). The pharmacokinetic studies involved the use of ^{3}H-HbV in which the lipid component (cholesterol) was radiolabeled with tritium. In addition, relationships between liver function and the pharmacokinetic parameters of HbV were examined.

MATERIALS AND METHODS

Preparation of HbV

HbVs were prepared under sterile conditions, as previously reported.[15] A hemoglobin solution was isolated and purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated hemoglobin (38 g/dL) contained 14.7 mM of pyridoxal 5′-phosphate (Sigma Chemical Co., St. Louis, Missouri) as an allosteric effector to regulate P_{50} to 25 to 28 torr. The lipid bilayer comprised a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG (NOF Corp., Tokyo, Japan) at a molar ratio of 5:5:1:0.03. The average particle diameter was 250 to 280 nm. HbVs were suspended in a physiological salt solution.

Preparation of CCRs Induced by CCl_4

All animal experiments were carried out in accordance with the guideline principles and procedures of Kumamoto University for the care and use of laboratory animals. Male Sprague–Dawley rats (48–51 g body weight; 3 weeks old; Kyudou Co., Kumamoto, Japan) were used in the study. All animals were maintained under conventional housing conditions, with food and water *ad libitum* in a temperature-controlled room with a 12-h dark/light cycle.

Sprague–Dawley rats were intraperitoneally injected with CCl_4 mixed with mineral oil at a dose of 400 mg/kg (volume of 0.45 mL/kg, CCl_4/mineral oil = 1:4) three times weekly for 56 days.

Assessment of CCRs Induced by CCl_4

One day after the last injection of CCl_4, six CCRs were anesthetized with ether. The CCRs were laparotomized to collect ascites, and the volume of ascites was measured. Subsequently, blood samples were collected from the inferior vena cava. Immediately, the ammonium concentration was measured. The concentration of ammonia was determined by the indophenol reaction, using an Ammonia-Test-Wako kit (Wako Pure Chemical Industries, Osaka, Japan). A part of blood was treated with citric sodium for the analysis of prothrombin time (PT). The remaining blood was centrifuged (3000 × *g*, 10 min) to obtain plasma for the analysis of albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GTP), total bilirubin (T-bilirubin), creatinine, and blood urea nitrogen (BUN). PT and all plasma parameters were analyzed by a commercial clinical testing laboratory (SRL, Tokyo, Japan). After collecting blood, the rats were laparotomized to be killed humanely with acute bleeding from the abdominal aorta and to obtain the liver for a histopathological study. The livers were fixed in 4% paraformaldehyde overnight.

The grade of cirrhosis severity in the CCR was classified by the Child–Pugh classification. The score of each test item was followed in accordance with the law,[16] and the sum of individual points were categorized as Child–Pugh grades A (5–6 points), B (7–9 points), and C (10–15 points).

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Histopathological Examination

The fixed livers were embedded in paraffin and sectioned into 5-μm slices, and the sections were stained with hematoxylin/eosin (H&E) and Elastica van Gieson (EVG) stains for the assessment of hepatic fibrosis as previously reported.[17] The area of hepatic fibrosis was assessed by means of image analysis software (Lumina Vision, Version 2.2, Mitani Co., Fukui, Japan; Image J, National Institutes of Health, Bethesda, Maryland). The fibrotic area was calculated as the mean of 30 randomly selected fields of vision in each liver section on the basis of the EVG stain.

Pharmacokinetic Experiments

^{3}H-HbV were prepared as previously reported[9] and mixed with 5% recombinant human serum albumin (Nipro Co., Osaka, Japan) to adjust the colloid osmotic pressure of the suspension to approximately 20 mmHg.[15]

Before the injection of ^{3}H-HbV, blood was collected and AST and ALT levels were determined using the commercial kit Transaminase CII Test Wako (AST and ALT) (Wako Pure Chemical Industries, Osaka, Japan). All of the CCRs were anesthetized with ether and received a single injection of ^{3}H-HbV suspension (1400 mg of Hb/kg). At each time point (3, 10, 30 min, 1, 3, 6, 12, 24, 48, and 72 h) after an injection of the ^{3}H-HbV suspension, blood was collected from the tail vein and plasma was isolated by centrifugation (3000 × g, 5 min). The plasma samples were also ultracentrifuged to collect intact HbV (50,000 × g, 30 min).[9] At 24 and 72 h after an injection of ^{3}H-HbV, the six animals were killed humanely and their livers and spleens were collected. Urine and feces were collected at fixed time points (3, 6, 12, 48, and 72 h) in a metabolic cage. The radioactivity was determined as previously reported.[9]

Carbon Clearance Measurement

The carbon clearance was determined using a previously described method, with minor modifications.[13] In a typical experiment, CCRs were anesthetized with ether. Polyethylene catheters (PE 50 tubing, outer diameter = 0.965 mm, and inner diameter = 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were then introduced into the left femoral vein both for infusion of a carbon particle solution and for blood collection. The carbon particle solution (Fount India Ink, Pelikan Co., Hannover, Germany) was infused at 10 mL/kg within 1 min. At 4, 10, 20, 30, 45, and 60 min later, about 100 μL of blood was 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 with a spectrophotometer (U-2900, HITACHI, Tokyo, Japan). The phagocyte index (K) was calculated using the following 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.

Data Analysis

A two-compartment model was used in the pharmacokinetic analyses. The total clearance (CL) and the area under the concentration curve (AUC) were calculated using the following equations: AUC = $(A/\alpha) + (B/\beta)$ and CL = Dose/AUC, where A, B and α, β are coefficients or exponents in the model equation. Each parameter was calculated by least-squares fitting by using MULTI.[18] Data are shown as means ± SD for the indicated number of animals. Overall differences between groups were determined by one-way of analysis of variance. The Spearman test was used for the correlation analyses. A probability value of $p < 0.05$ was considered as statistically significant.

RESULTS

Biochemical Analysis

One day after the last injection of CCl_4, the average body weight of the control rats and the CCRs were 469 ± 35 and 318 ± 54 g, respectively ($p < 0.01$). To assess the severity of hepatic impairment, we measured the AST, ALT, γ-GTP, T-bilirubin, plasma albumin, and ammonia levels in blood. As a result, the AST and ALT levels were significantly increased in the CCRs compared with the normal rats (AST: 82.8 ± 10.7 and 519 ± 216 IU/L, $p < 0.01$; ALT: 46.4 ± 5.6 and 157 ± 37 IU/L, $p < 0.01$, for normal rats and CCRs, respectively). γ-GTP levels were significantly increased by more than twofold in the CCRs (3.0 ± 0.1 and 9.5 ± 6.1 IU/L, $p < 0.01$, for normal rats and CCRs, respectively). The plasma T-bilirubin and ammonium levels in blood were also significantly increased, and the albumin levels were significantly decreased in the CCRs (T-bilirubin: 0.92 ± 0.28 and 10.5 ± 13.6 μmol/L, $p < 0.01$; ammonium: 81.6 ± 24.4 and 252 ± 93 mg/dL, $p < 0.01$; albumin: 41.8 ± 1.6 and 30.8 ± 2.5 g/L, $p < 0.01$, for normal rats and CCRs, respectively).

Histological Observation and Quantification of the Hepatic Fibrotic Area

Figure 1 shows histological findings for livers stained with H&E and EVG. From the H&E staining data, hepatic damage was clearly observed in the CCRs but not in the normal rats (Figs. 1a and 1b). In addition, fibrosis was assessed on the basis of EVG staining, which is specific for the presence of fibrosis.[17] As shown in Figures 1c and 1d, fibrosis was observed in the CCRs but not in the normal rats. The area of the fibrosis in the CCRs was significantly increased to approximately 15% of the area of vision in each CCR (Fig. 1e).

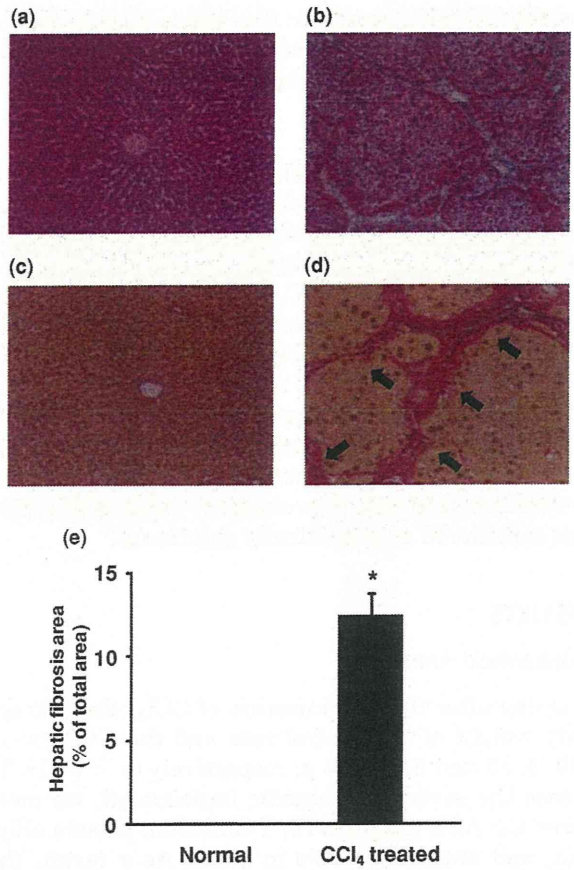

Figure 1. Light micrographs of livers stained with H&E and EVG stains. Normal (a) and CCl_4 treated (b) groups (100×) stained with H&E. Normal (c) and CCl_4 treated (d) groups (100×) stained with EVG. The arrows indicate fibrosis areas. (e) Hepatic fibrosis area in the normal ($n = 6$) and CCl_4 treated ($n = 6$) groups. The fibrotic area was calculated as the mean of 30 randomly selected fields of vision in each liver section, based on EVG staining. Data are expressed as mean ± SD. $^*p < 0.01$.

Child–Pugh Assessment

The CCRs used in this study were further evaluated by Child–Pugh classification (Table 1). Encephalopathy, such as asterixis and abnormal behavior, was not observed during experiments. The plasma T-bilirubin level and PT were 10.5 ± 13.6 μmol/L and 51.6 ± 6.6%, respectively. These values correspond to a score of 1

Table 1. Child–Pugh Assessment

		Score
T-bilirubin (μmol/L)	10.5 ± 13.6	1
Albumin (g/L)	30.8 ± 2.5	2
PT (%)	51.6 ± 6.6	2
Encephalopathy	None	1
Ascites (mL)	24.1 ± 17.1	3
Total		9

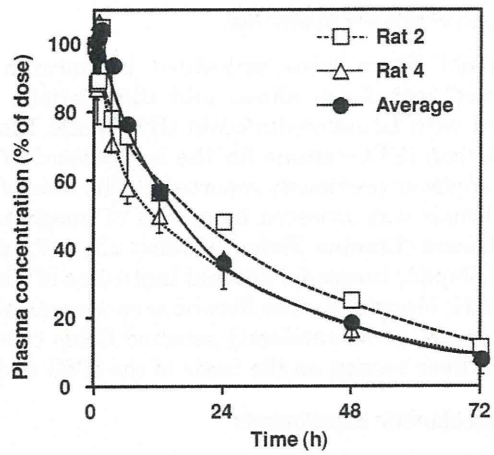

Figure 2. Time course profile for plasma levels of ^{3}H-HbV after administration to rat 2 (open square) and rat 4 (open triangle). The closed circle was represented mean ± SD ($n = 8$).

or 2 in the Child–Pugh classification system. The albumin level was 30.8 ± 2.5 g/L, which corresponds to a score of 2. The score for ascites in humans allows patients to be classified as *absent*, *moderate*, or *abundant*. In this study, the ascites in the CCRs were evaluated by measuring the ascites volume. The ascite volume in the CCRs was 24.1 ± 17.1 mL, a volume equivalent to approximately 10% of the body weight. Moreover, ascites was reproduced within 2 days after their removal. On the basis of these data, we categorized the ascites in our CCRs as *moderate* or *abundant* in the Child–Pugh classification. In addition, no encephalopathy was observed in the CCRs. Therefore, the score, corresponding to the sum of individual points, allows the CCRs to be categorized as Child–Pugh grade B.

Relationships Between Hepatic Injury and the Pharmacokinetic Parameters of HbV

To investigate the relationships between hepatic injury and the pharmacokinetics of HbV, we examined the pharmacokinetics of HbV in the CCRs. Figure 2 showed the time course of HbV concentration in plasma after administration of ^{3}H-HbV at a dose of 1400 mg of Hb/kg to the CCRs. The plasma concentration of HbV was varied widely among individuals. As shown in Figure 3, the CL and the AUC values for HbV, as calculated from the time course of the plasma concentration curve, were plotted against the plasma AST levels. As shown in Figure 3, CL was negatively correlated with plasma AST levels ($r = 0.81$, $p = 0.007$), whereas the AUC was positively correlated with plasma AST levels ($r = 0.81$, $p = 0.007$). However, CL and AUC were not significantly correlated with plasma ALT levels (CL: $r = 0.19$, $p = 0.44$; and AUC: $r = 7 \times 10^{-3}$, $p = 0.49$) because the ALT values

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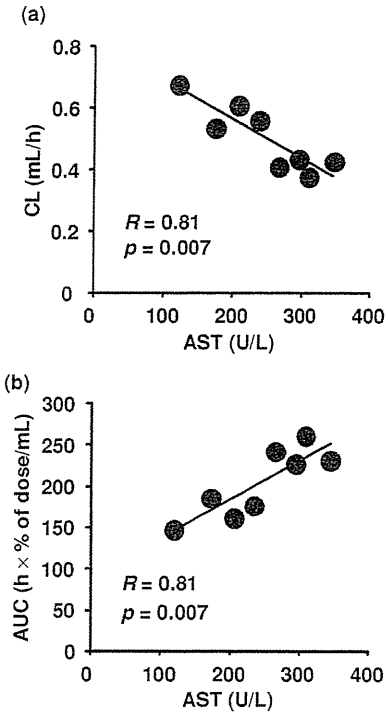

Figure 3. Relationship between plasma aspartate aminotransferase (AST) levels and (a) total clearance (CL) or (b) area under the concentration curve (AUC) for HbV. The linear regression was calculated using the least-squares method (a: $y = -0.0012x + 0.814$, $r = 0.81$, $p = 0.007$; b: $y = 0.4697x + 89.3$, $r = 0.81$, $p = 0.007$).

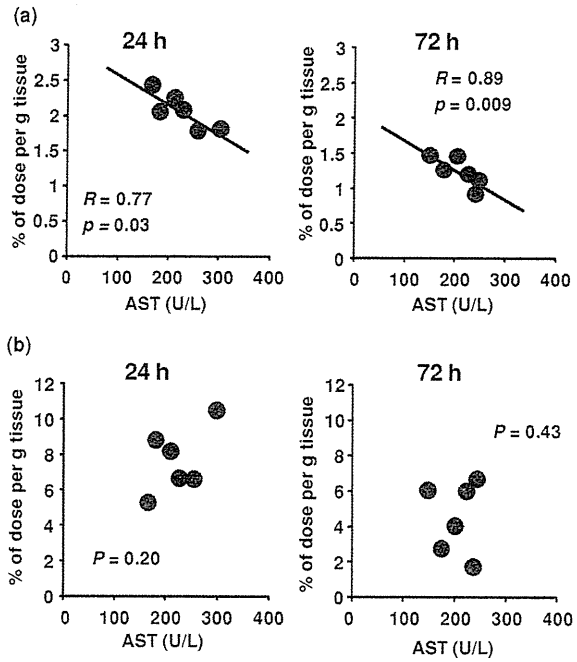

Figure 4. Relationship between plasma aspartate aminotransferase (AST) levels and (a) hepatic or (b) splenic distribution of HbV at 24 or 72 h after the administration of ^{3}H-HbV. The linear regression was calculated using the least-squares method (a: 24 h, $y = -0.0042x + 2.99$, $r = 0.77$, $p = 0.03$; 72 h, $y = -0.0042x + 2.09$, $r = 0.89$, $p = 0.009$).

were essentially unchanged among individual CCRs (data not shown). These data indicate that HbV elimination from the circulation is significantly dependent on the severity of hepatic impairment.

Relationships Between Hepatic Impairment and the Tissue Distribution of HbV

Because the liver and spleen are the major distribution and metabolic tissue for HbV,[9] the effect of hepatic impairment on the hepatic and splenic distribution of HbV was examined. In Figures 4a and 4b, the hepatic and splenic distribution of HbV at 24 or 72 h after ^{3}H-HbV injection was plotted against the plasma AST level. As a result, the hepatic distribution at both 24 and 72 h was well negatively correlated with AST levels (Fig. 4a) (24 h: $r = 0.77$, $p = 0.03$; 72 h: $r = 0.89$, $p = 0.009$), whereas the splenic distribution was not (Fig. 4b). On the other hand, the hepatic and splenic distribution was not correlated with plasma ALT levels (data not shown). These data clearly indicate that the alterations in the pharmacokinetic parameters (Fig. 3) with increased AST levels are associated with the decrease in the hepatic distribution of HbV.

Relationships Between Hepatic Impairment and Phagocyte Activity

It was previously reported that HbV is scavenged and degraded by the mononuclear phagocyte system (MPS), such as KCs and red pulp zone splenocytes.[9,13] Therefore, we hypothesized that phagocyte activity, especially phagocytes in KCs, are related to the changes in the pharmacokinetic properties of HbV, as shown in Figures 3 and 4. To evaluate the relationship between phagocyte activity and hepatic impairment, carbon clearance in the CCRs was determined. Figure 5 shows a plot of the phagocyte index (K) against plasma AST levels. Carbon clearance was negatively correlated with AST levels (Fig. 5) ($r = 0.81$, $p = 0.047$). However, it was not significantly correlated with plasma ALT levels ($r = 0.24$, $p = 0.35$).

Relationships Between Hepatic Impairment and the Urinary or Fecal Excretion of HbV

It was previously reported that the pathway for the excretion of cefmetazole, an antibiotic agent, was altered from the fecal to the urinary pathway in the CCRs.[14] In addition, we previously reported that in normal rats, the outer lipid component, especially

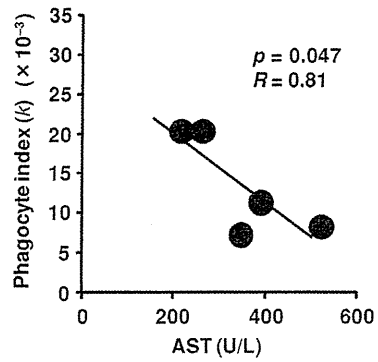

Figure 5. Relationship between plasma aspartate aminotransferase (AST) levels and phagocyte index (K). The carbon clearance was estimated, and K was calculated from the clearance of carbon particles. The linear regression was calculated using the least-squares method ($y = -0.00004x + 0.0289$, $r = 0.81$, $p = 0.047$).

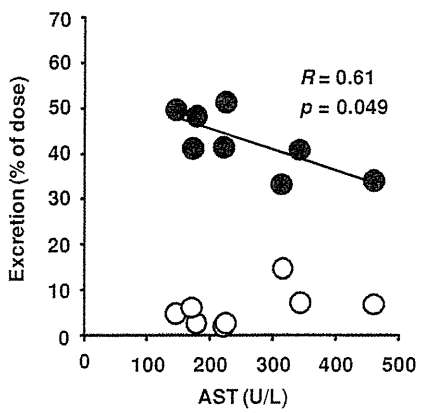

Figure 6. Relationship between plasma aspartate aminotransferase (AST) levels and excretion via the feces (closed circle) or urine (open circle). The linear regression was calculated using the least-squares method (feces: $y = -0.0464x + 54.364$, $r = 0.61$, $p = 0.049$).

cholesterol, is mainly eliminated by the feces via biliary excretion.[9] Therefore, the effect of hepatic impairment on the excretion of the lipid component (cholesterol) of HbV was examined. The lipid component of HbV was largely excreted via the fecal pathway in the CCRs (45.9 ± 10.2% of dose), and urinary excretion was virtually undetectable (5.6 ± 4.5% of dose) at 72 h after ^{3}H-HbV injection. Figure 6 shows the relationship between the 72-h cumulative excretion of the lipid component in feces or urine and the plasma AST levels. The excretion of the lipid component in feces was negatively correlated with AST levels ($r = 0.61$, $p = 0.049$), whereas that in urine was not ($r = 0.47$, $p = 0.12$). Neither excretion in feces nor excretion in urine was correlated with plasma ALT levels (feces: $r = 0.34$, $p = 0.20$; urine: $r = 0.068$, $p = 0.43$).

DISCUSSION

The findings reported herein demonstrate that hepatic impairment altered the pharmacokinetic properties of HbV, as evidenced by a reduction in CL, hepatic distribution, and fecal excretion by a reduction in KC phagocyte activity and damage to parenchymal cells. In addition, these parameters were well correlated with the plasma AST levels but were not significantly correlated with plasma ALT levels.

Sotaniemi et al.[19] reported that the CL of antipyrine is affected by chronic liver failure and that this is caused by a decrease in cytochrome P450 content in the liver. Although HbV is not metabolized by cytochrome P450, KCs and splenic macrophages mainly scavenge and metabolize HbV.[9,13] Therefore, the possibility that the pharmacokinetics of HbV is also affected by hepatic impairment cannot be excluded. The findings of this study clearly indicate that pharmacokinetic parameters, such as CL and AUC, of HbV in CCRs are well correlated with changes in plasma AST levels (Fig. 3). In addition, the hepatic distribution of HbV was negatively correlated with plasma AST levels, but this was not found for the spleen (Fig. 4). These results demonstrate that the pharmacokinetic properties of HbV are significantly influenced by a reduction in liver function. Moreover, the carbon clearance, which serves as a measure of phagocyte activity, was also negatively correlated with plasma AST levels (Fig. 5). According to a previous report, the KCs phagocytosed more than 90% of the injected carbon particles.[20] Therefore, present results strongly suggest that the change in HbV pharmacokinetic properties was likely dependent on a decrease in phagocyte activity, especially KCs, in CCRs.

Several investigators previously reported that the number and activity of KCs increased during CCl_4 administration to rats.[21,22] However, in our model, we found that phagocyte activity of KCs was not increased by CCl_4 treatment. Such contradictory findings between our and their study could be due to the differences in the experimental conditions employed. In this study, we measured carbon clearance to evaluate phagocyte activity, especially KCs, under *in vivo* conditions. In contrast, they collected KCs from whole livers of CCRs and evaluated the number and activity of the KCs *in vitro* studies. However, it was not clear whether the purified KCs from CCRs had the ability to scavenge invaders under *in vivo* conditions. Chronic liver cirrhosis would lead to disordered structural and functional changes of hepatocytes, and hepatic hemodynamics would subsequently become deteriorated. In addition, the population and localization of hepatocytes, such as parenchymal, sinusoidal endothelial, Kupffer, and Ito cells, would also change. Therefore, it is necessary to consider the changes in the hemodynamics and the pathology of the liver to

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properly interpret the data. In our model, the area of the fibrosis was approximately 15% of the visible area in the liver (Figs. 1d and 1e), which corresponds to Child–Pugh grade B (Table 1). Therefore, HbV and carbon particles reaching KCs would be decreased under such hepatic pathological and hemodynamic conditions in our model, as they would occur in normal conditions. In fact, several previous reports on hemodynamic and pathological changes in rats that had been treated with CCl_4 support this hypothesis: (a) intrahepatic blood flow is decreased because of structural and functional changes that occur in chronic liver cirrhosis[23]; (b) the pathological process of developing cirrhosis represents an almost inconceivably complex network of interacting cells, and the loss of sinusoidal density is observed in CCRs[24]; (c) the KCs appeared to aggregate primarily along the fibrotic septa[25]; and (d) the amount of fluorescein isothiocyanate–labeled dextran (molecular weight = 150,000) reaching the sinusoid area was negligible.[24]

Another interesting finding in the present study is that the suppression of fecal excretion of cholesterol derived from HbV membranes was accompanied by an increase in plasma AST levels (Fig. 6). Our previous study demonstrated that HbV cholesterol behaves similar to endogenous cholesterol after being metabolized by the MPS.[9] Generally, the excess amount of endogenous cholesterol is converted into high-density lipoprotein cholesterol esters, which are directly taken up by hepatic parenchymal cells and subsequently efficiently coupled to bile acid formation and secreted via the feces. When hepatic parenchymal cells are damaged, the hepatocellular excretory function (i.e., bile flow) decreases in CCRs.[24] Thus, the biliary lipid secretion of cholesterol, bile acids, and phospholipids is markedly decreased in chronic liver disease.[26] These data support the conclusion that hepatic impairment also induced a reduction in the excretion of cholesterol derived from HbV.

Such a suppressed elimination of HbV components may have an impact on their tissue accumulation. Actually, perfluorocarbon, which has also been developed for use as an artificial oxygen carrier, was excluded from the possible list of candidates for artificial oxygen carriers because it remained inside the body, especially the lungs, for 1 year or longer.[27] In this study, ^3H nearly completely disappeared from organs after 14 days, even in the CCRs (data not shown). In addition, the levels of other components of HbV, such as phospholipids and ferric species, temporarily increased after the administration of HbV at a dose of 1400 mg of Hb/kg in the CCRs but recovered to the baseline levels within 14 days (submitted in a separate paper). Similar data were obtained for the same experiment in normal rats.[9,28] Therefore, HbV exhibits an acceptable metabolic and excretion performance even under the condition of chronic cirrhosis, suggesting that it can be used safely in conditions of hepatic impairment.

In addition, in this study, a correlation between the clearance and hepatic distribution of HbV and plasma AST levels was found but not for the case of plasma ALT levels. It appears that a biological reason would exist for the lack of variation in plasma ALT levels among individual rats. In addition, generally, plasma AST levels have precedence over plasma ALT levels in hepatic cirrhosis. Because the CCRs used in this study had moderate or severe cirrhosis (Child–Pugh grade B), some of the pharmacokinetic data in this study would be correlated with only plasma AST levels. Furthermore, these liner relationships between the AST level and pharmacokinetic data were not observed when the results from the normal rats fit into those from the CCRs (data not shown). The similar results was reported by Okumura et al.[14] Therefore, these correlations seem to be applicable only to the condition of liver impairment.

From the viewpoint of future clinical applications of HbV, it is important to determine whether the optimal dose of HbV should be adjusted in the patients with hepatic impairment. We previously reported that the half-life of HbV at a dose of 1400 mg of Hb/kg in humans appears to be 3 to 4 days,[11] and this half-life indicates that it could be used as a temporary oxygen carrier until a blood transfusion or until autologous blood is recovered after a massive hemorrhage. In the present study, the reduction in plasma CL of HbV was strongly dependent on an increase in plasma AST levels, indicating that the half-life of HbV is increased with accompanying liver damage. This leads to the conclusion that the infusion dose of HbV may be adjusted on the basis of plasma AST levels in cases of liver failure. However, it is premature to conclude this fact on the basis of limited data that are currently available. In the present study, we examined only the pharmacokinetics of HbV in a moderately damaged CCR (Child–Pugh grade B) induced by CCl_4. Because the pathology of liver failure is not simple and differences in the extent of liver damage exist, such as fibrosis and injury, it will be necessary to investigate the pharmacokinetics of HbV and its dependence upon plasma AST levels in various CCR models, for example, in concanavalin A- and acetaminophen-induced models. In addition, we did not test for other parameters of liver impairment, such as bile flow, albumin synthesis, and coagulation factors, with respect to the correlation with the pharmacokinetic parameters of HbV. This would provide more information on relationships between the pharmacokinetics of the liposome preparation and hepatic impairment. This issue deserves further study. Moreover, because carbon clearance can clearly demonstrate the phagocyte functions, further experiments might be needed to strongly clarify the relationship

between varied clearance in chronic condition and the impaired phagocytosis-dependent uptake in the KCs. To further demonstrate this, the pharmacokinetic experiment using CCRs pretreated with $GdCl_3$, which specifically inhibits the KCs, will be needed. Finally, because the pharmacodynamics of HbV is related to pharmacokinetics, it will also be important to clarify the relationship between the pharmacokinetics and pharmacodynamics of HbV in CCRs for determining an optimal dosage regimen.

CONCLUSION

The present study clearly demonstrates that the pharmacokinetics of HbV is altered by hepatic impairment and the changes in pharmacokinetic parameters were well correlated with the plasma AST levels. This could be due to the decrease in KC function induced by hepatic impairment. To our knowledge, this is the first report of the relationships between the pharmacokinetics of a liposome preparation and hepatic impairment. Therefore, these data will be useful in estimating the pharmacokinetics of not only HbV but also other liposome preparations in hepatic impairment.

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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.

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-

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

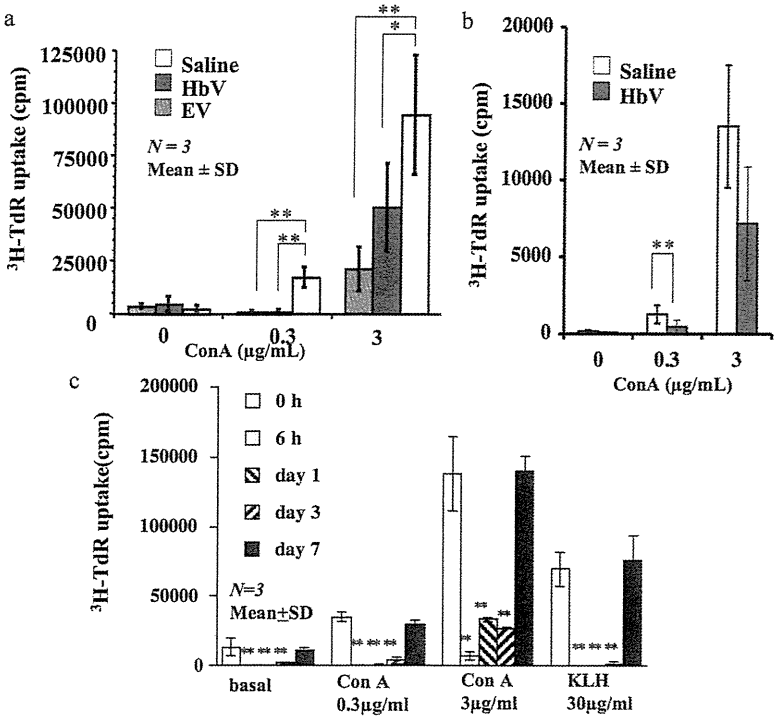


Fig. 1. Effect of HbV and empty vesicles on proliferation of Con A-stimulated rat splenic T cells. a, spleens were excised from rats 24 h after injection of HbV, EV, or saline (control); splenic single cells were stimulated with Con A. Both HbV and EV induced significant suppression of the proliferative 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

a

Production of IL-2 by splenocytes (pg/ml) *(N=3)*

Load	Con A(μg/ml) 0	0.3	
saline	0	100±61	n.s.
HbV	0	111±30	

b

Production of NO by splenocytes (μM)

load	mitogen(-) inhibitor(-)	Con A (0.3 μg/ml) 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

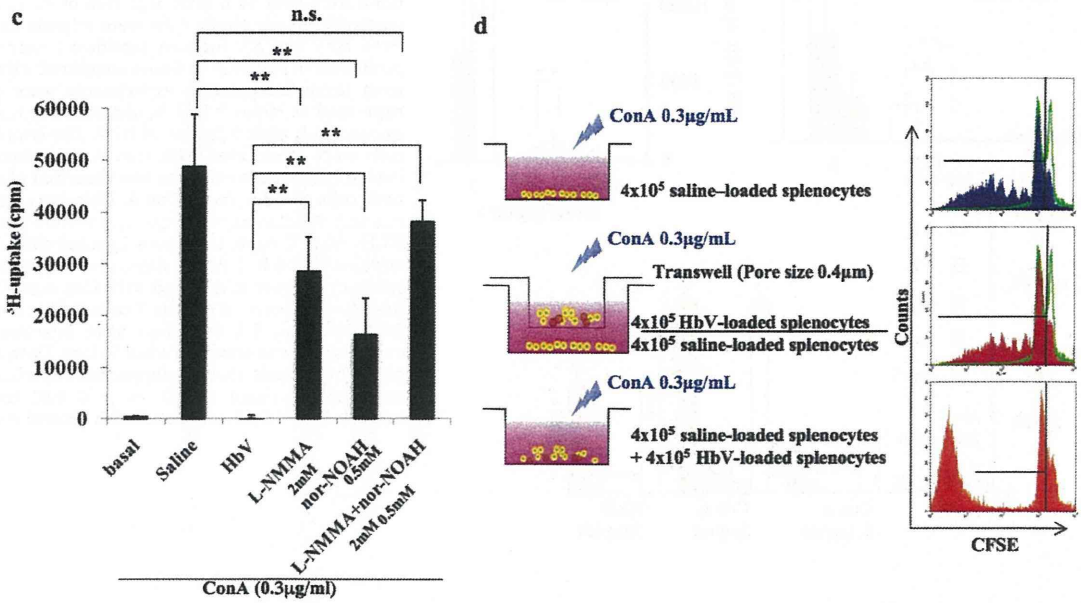


Fig. 2. Analysis of the mechanism of T cell suppression. a, each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) for 48 h; then supernatant was collected and IL-2 was measured as described under *Materials and Methods*. Data were collected from three independent 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 (4×10^6). 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.

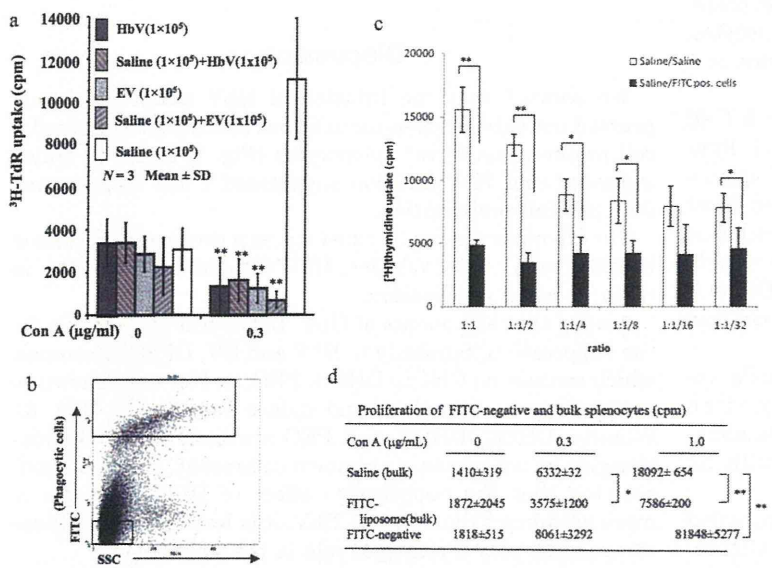
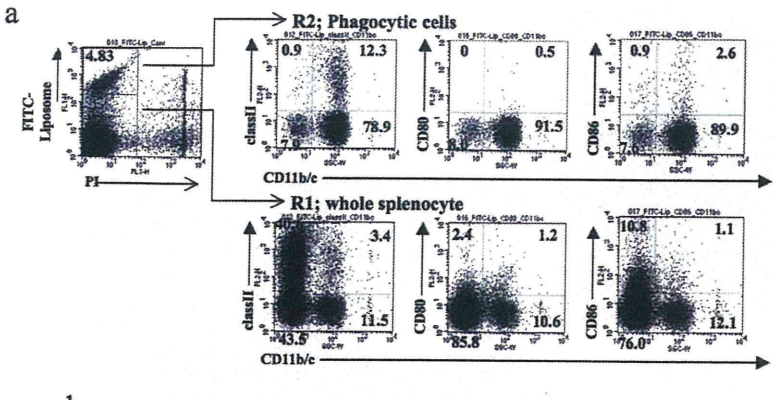


Fig. 3. Immune suppressor cells are induced after infusion of HbV (or empty liposomes). a, HbV- or EV-loaded bulk spleen cells (1×10^5) were mixed with control bulk spleen cells (1×10^5) and stimulated with Con A (0.3 μg/ml). T cell proliferation was clearly suppressed. **, $p < 0.01$. Data are representative 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



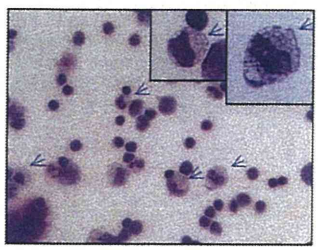


Fig. 4. Phenotypic and morphological analyses of FITC-liposome phagocytized cells. a, splenocytes derived from FITC-liposome-loaded rat spleen were analyzed. Top, FITC-positive cells were gated in R2. Bottom, whole splenocytes were gated in R1. Subsequently, the gated cells were analyzed for the indicated 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.

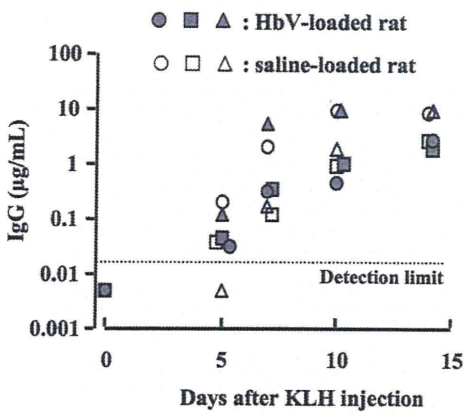


Fig. 5. Effect of HbV on primary antibody response. Primary antibody response to KLH was evaluated as described under *Materials and Methods*. The kinetics of the primary antibody response of three rats preloaded with HbV was shown to be comparable with that of three rats preloaded with saline.

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

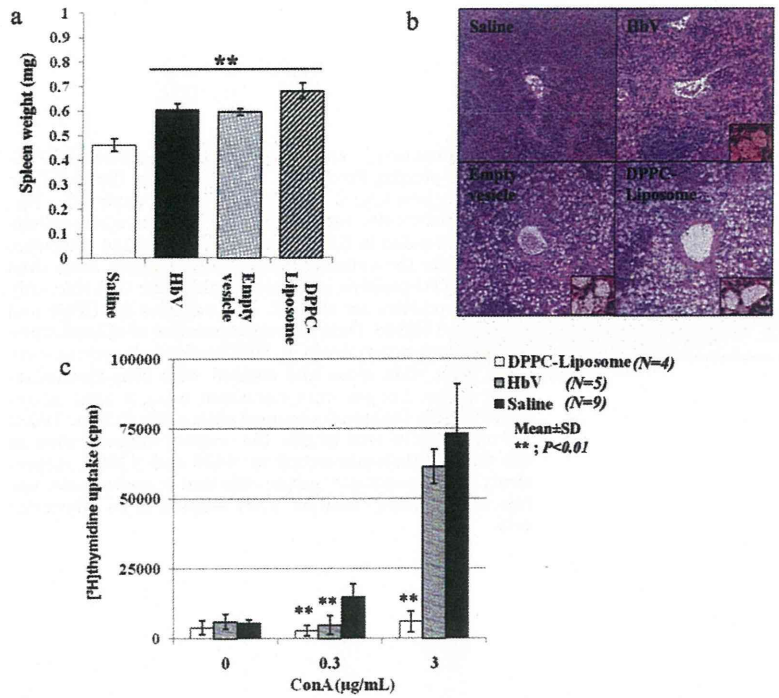


Fig. 6. Effect of DPPC-liposome on immune suppression. HbV, EV, DPPC-liposome, or saline was injected intravenously. The spleen was excised 18 h later. a, the spleen mass increased more after injection of HbV, EV, and DPPC-liposomes than after injection of saline alone. Data are expressed as mean ± S.D. (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.

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.

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-