gated K^+ channel. Furthermore, the CSE activities have been reported to be altered under disease conditions; the activity in the liver is reduced in patients with liver cirrhosis and in those exposed to surgical insults or acquired immune deficiency syndrome (8, 10, 20). On the other hand, experimental models of vitamin B_6 deficiency or streptozotocin-induced diabetes revealed alterations in CSE in the liver under these disease conditions (6, 15). Until now, however, effects of such alterations in the activities on organ functions and roles of H_2S under these circumstances have not been fully investigated yet.

This study was designed to focus first on differences in contribution of CSE to tissue H_2S generation; the data indicated that the liver constitutes one of the largest organ components for the gas generation in the body. Based on this result, we further attempted to examine if H_2S derived from the enzyme could play a role in the regulation of hepatobiliary function. The current results first provided evidence that the liver utilizes this gaseous substance as a modulatory determinant of biliary bicarbonate excretion.

MATERIALS AND METHODS

In vivo *and* ex vivo *determination of bile constituents*

The experimental protocols herein described were approved by our institutional guidelines provided by the Animal Care Committee of Keio University School of Medicine. Male Wistar rats weighing 220–260 g (CLEA Japan, Tokyo, Japan) were allowed free access to laboratory chow and tap water, and were fasted for 24 h prior to experiments. As described elsewhere, rats were anesthetized with an intramuscular injection of pentobarbital sodium at 50 mg/kg, and their common bile ducts were cannulated to collect bile samples. Bile output was monitored *in vivo* according to our previous method (7). When necessary, livers of these rats were perfused *ex vivo* with the oxygenated Krebs–Henseleit buffer at a constant flow rate of 4 ml/min/g of liver in a single-pass mode (14). Bile samples collected through a cannulation were used to determine concentrations of total bile salts, phospholipids, pH values, and bicarbonate (HCO_3^-) according to previous methods described elsewhere (7, 14).

Experimental protocols

Propargylglycine (PPG) was used as a potent inhibitor of CSE. PPG was dissolved in physiological saline as a vehicle and administered intraperitoneally at a dose of 1.5 mmol/kg of body weight at 4 h prior to the preparation for bile duct cannulation. Bile was collected every 10 min until the end of experiments according to our previous method (7). In the case of experiments using the *ex vivo* perfused preparation, livers were excised from the PPG-treated rats and perfused with the Krebs–Henseleit buffer containing 300 μmol/L PPG to avoid a possible reduction of the enzyme blockade due to elimination of the reagent from the system. To examine effects of the intraperitoneal injection of the CSE inhibitor on endogenous H_2S generation, we determined tissue contents of the gas *in vivo*. Livers were excised and snap-frozen at 4 h after the treatment with PPG or vehicle, and the samples were minced with 0.1 *N* NaOH to remove proteins. Amounts of H_2S in the liver tissues were determined by gas chromatography according to previous methods described elsewhere (4). In separate sets of experiments, bile output was monitored every 10 min after establishment of the bile duct cannulation, and concentrations and fluxes of bile constituents were compared between the control and PPG-treated groups. To examine if effects of PPG are attributable to a reduction of the reaction product of CSE such as H_2S, we examined effects of supplementation of NaHS, a soluble donor of the gas at desired concentrations, in the buffer for the *ex vivo* perfusion system. As a control set of the experiments, we compared effects of the same concentrations of *N*-acetylcysteine (NAC), a cysteine donor. In experiments using isolated *ex vivo* perfused livers, sodium taurocholate was added to the buffer at desired concentrations in a range between 0 and 30 μmol/L. Using data collected from these experiments, the bile acid-independent fraction of bile output was determined by plotting bile output as a function of biliary output of bile salts in the samples: the value of the output at the *y*-intercept (zero concentration of bile salts) was regarded as the bile acid-independent fraction (2).

Immunohistochemistry

Liver tissues also served as samples for immunohistochemistry. An anti-CSE antibody was prepared by immunization to rabbit of the C-terminal peptide CYGGTNRYFRRVASE, the sequence of which is identical to that of the rat enzyme. The antibody was purified from the antiserum using affinity chromatography as described elsewhere (3). The specificity of the antibody was confirmed by western blot analyses. For immunohistochemistry, rat livers were removed to prepare OCT compound-embedded frozen sections (7 μm). The sections were immunostained with the anti-CSE antibody using the Vectastain ABC kit (Vector Laboratories), as previously described (5). Semiserial sections were stained with the anti-CSE antibody or with the anti-rat keratin 19 monoclonal antibody (MAB1675; Chemicon, Temecula, CA, U.S.A.) to examine colocalization of the enzyme with biliary epithelium and hepatocellular bile canaliculi, when necessary.

Statistical analyses

The statistical significance of data among different experimental groups was determined by one-way ANOVA and Fisher's multiple comparison test. $p < 0.05$ was considered significant.

RESULTS

Liver constitutes the largest organ component for CSE-derived H_2S generation

Figure 1 illustrates tissue contents of H_2S in different organs. The control liver treated with vehicle contained ~80 nmol/g of tissue of the gas (Fig. 1A). Livers from rats pretreated with 1.5 mmol/kg PPG, an inhibitor of CSE, suppressed the constitutive levels of the gas by 50%. The dose of PPG used in this experiment appeared to be sufficient enough to block the enzyme, as indicated by dose responses of the H_2S contents as a function of doses of the inhibitor (Fig. 1B). When the tissue gas

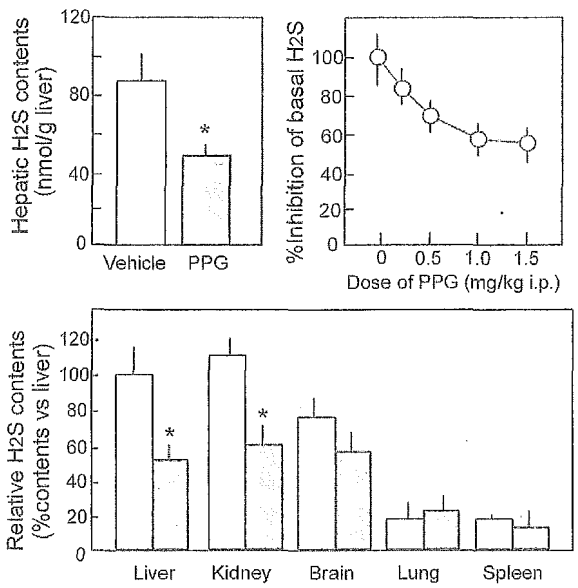

FIG. 1. Effects of administration of PPG, an inhibitor of CSE, on tissue contents of H_2S *in vivo*. (A) The effects of the PPG administration on hepatic H_2S contents. PPG was intraperitoneally injected at 1.5 mmol/kg at 4 h prior to the experiments. Data indicate means ± SE of more than eight separate experiments. $^*p < 0.05$ as compared with the vehicle-treated control group. (B) Dose-dependent effects of PPG on the basal H_2S contents in rat livers. (C) Differences in the sensitivity to PPG administration among organs. Open and filled bars represent the tissue H_2S contents in the vehicle- and PPG-treated groups, respectively. Data indicate means ± SE of four separate experiments. $^*p < 0.05$ as compared with the vehicle-treated control group.

contents were compared among different organs (Fig. 1C), liver appeared to constitute the largest organ component for endogenous H_2S production; the level was comparable to that measured in the kidney and 1.5-fold greater than that in the brain. So far as judged by sensitivity to PPG, the gas generation in the liver and kidney depended largely on CSE, whereas that in other organs, such as brain, lung, and spleen, seemed CSE-independent; the finding is consistent with previous observations in mouse brain tissues where CBS constitutes a major source for the gas generation (1).

CSE-derived H_2S is a determinant of the basal bile output and biliary HCO_3^- excretion

Figure 2 demonstrates protein expression of CSE in rat liver tissues. Western blot analyses indicated that the purified polyclonal antibody used in this study specifically recognized the enzyme at 40 kDa (Fig. 2A). Immunohistochemistry using the same antibody revealed that the most intense reactivities were seen in periductal regions of portal triads, whereas walls of hepatic arterial walls and terminal portal veins displayed little reactivities, if any. In addition, a modest expression of CSE was notable in hepatocytes, indicating intralobular homogeneity in its expression (Fig. 2B), whereas nonspecific IgG did not stain the slice (Fig. 2C). Figure 2D and E illustrates semiserial sections stained with the anti-CSE and anti-keratin 19 antibodies, respectively. As seen, cytokelatin-positive ductular structures connecting to bile canalicular networks near the portal triad exhibited notable CSE expression, whereas an artery adjacent to the portal vessel did not display evident immunoreactivities. The staining disappeared when the anti-CSE antibody was absorbed by adding the antigen peptide (data not shown).

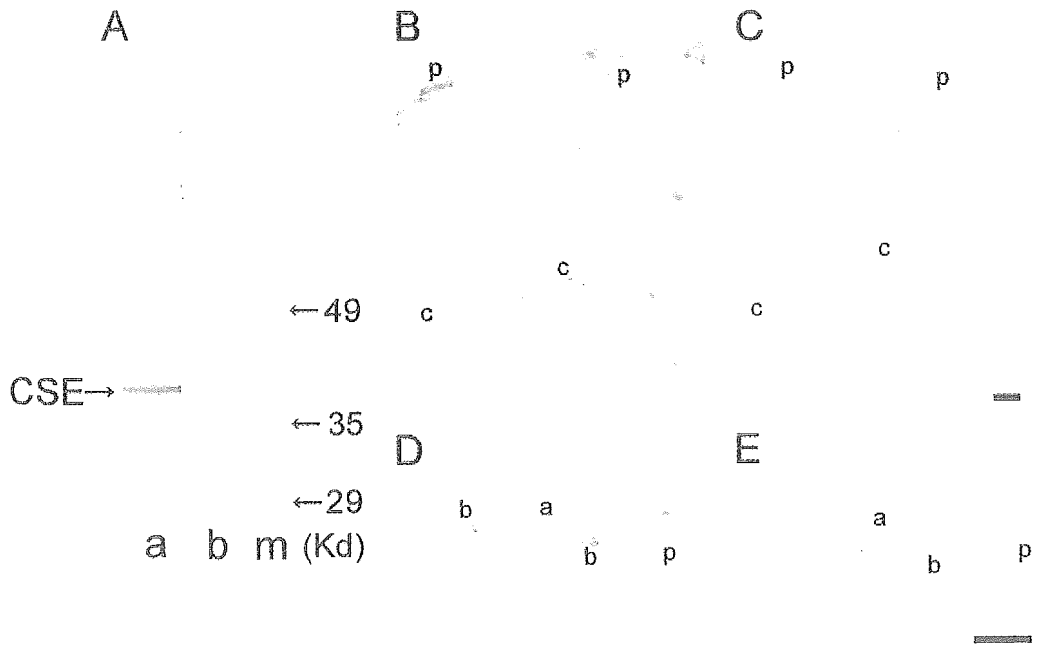

FIG. 2. Expression of CSE in the rat liver. (A) western blot analyses using the anti-rat CSE antiserum (lane a) and the affinity column-purified antibody (lane b). m: molecular markers. Note a single band in lane b. (B and C) Intralobular distribution of CSE in the rat liver stained with the purified anti-CSE antibody and with nonspecific chicken IgG, respectively. p and c: portal and central venules. (D and E) High magnification of a representative slice stained with the anti-CSE antibody and with the anti-keratin 19 antibody, respectively. a and b: artery and biliary duct. Bars = 50 μm.

TABLE 1. EFFECTS OF BLOCKADE OF CSE BY PPG ON BASAL BILE OUTPUT AND BILIARY HCO_3^- EXCRETION

Groups	*Basal bile output (μl/min/g of liver)*	*Biliary HCO_3^- concentration (mmol/L)*
Vehicle ($n = 6$)	1.73 ± 0.09	27.9 ± 1.2
PPG ($n = 6$)	2.11 ± 0.05*	33.0 ± 0.7*

*$p < 0.05$ as compared with the vehicle-treated control group.

We determined the effects of systemic administration of PPG on bile output and biliary constituents *in vivo* according to the identical protocol used in Fig. 1. As shown in Table 1, the PPG administration significantly stimulated basal bile output by 15%. The biliary concentration of HCO_3^- was also significantly elevated in the PPG-treated group. As PPG inhibits CSE and could not only reduce endogenous H_2S, but also modify cysteine metabolism, it is necessary to examine the direct effects of exogenous H_2S administration on hepatobiliary function. However, such experiments were difficult, because the administration of NaHS, the H_2S-donating reagent, is known to change systemic blood pressure *in vivo* through its vasorelaxing action (22). We thus used livers perfused *ex vivo* with the taurocholate-free Krebs solution to prove roles of CSE-derived H_2S in the basal bile excretion.

As illustrated in Fig. 3, the hepatic vascular resistance was comparable among four groups tested (*e.g.*, vehicle, PPG, PPG + NaHS, and PPG + NAC). Under these circumstances, the basal bile output was significantly elevated by 20% in perfused livers of the PPG-treated rats as compared with those treated with vehicle. This response was slightly greater than that observed in the experiments *in vivo* (Table 1), presumably because the perfusion of the organ was carried out under cholate-free conditions, as discussed later in Results. The choleretic response elicited by the PPG treatment was repressed by coperfusion of NaHS at 30 μmol/L, the concentration being comparable to the PPG-sensitive fraction of the gas generation. On the other hand, coperfusion of the same concentration of NAC, a reagent entering cells to yield cysteine, did not alter the CSE-elicited choleretic response. Like the aforementioned observations *in vivo* (Table 1), the PPG treatment significantly enhanced biliary HCO_3^- concentrations, and coperfusion of 30 μmol/L NaHS completely attenuated the changes in the perfused rat livers. On the other hand, the NAC coperfusion did not repress the PPG-induced elevation of the HCO_3^- concentration (Fig. 3B).

As HCO_3^- serves as a putative constituent yielding the driving force for bile formation, we determined if the bile acid-independent bile formation is elevated in livers of the PPG-treated groups. As seen in Fig. 4, where the output was plotted as a function of biliary fluxes of bile salts, the *y*-intercept of the line for the PPG-pretreated groups became markedly decreased and dissociated from that for the control groups. The difference between the two groups became smaller with increasing fluxes of bile salts, but the difference was still evident when the flux of bile salts reached the physiologic levels (70 nmol/min/g of liver). Such a dependency of the PPG effect on bile salts was consistent with the current data indicating differences in the choleretic responses between *in vivo*

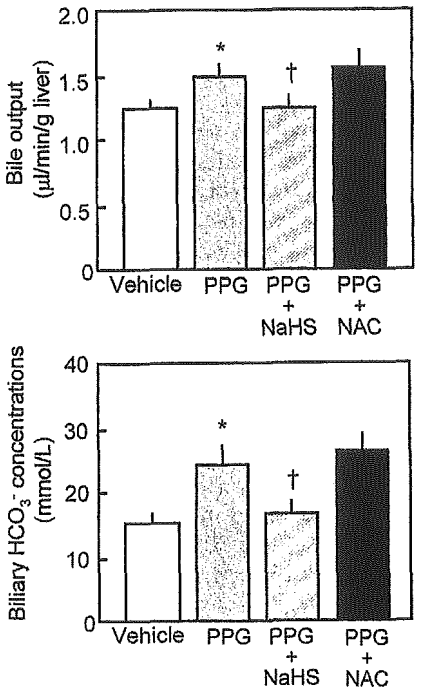

FIG. 3. Effects of the CSE blockade by PPG and supplementation of NaHS on the basal bile output and biliary HCO_3^- concentration in *ex vivo* perfused rat livers. PPG at 1.5 mmol/kg was administered *in vivo* intraperitoneally at 4 h prior to the isolation of the perfused liver. Either NaHS or NAC was perfused *ex vivo* into the liver at a concentration of 30 μmol/L, when necessary. Data indicate means ± SE of seven to nine separate experiments in each group. *$p < 0.05$ as compared with the vehicle-treated group; †$p < 0.05$ versus the PPG-treated group.

(Table 1) and *ex vivo* (Fig. 3) perfused livers. We further investigated whether biliary output of glutathione, another major constituent for bile acid-independent bile formation, could also be elevated under the blockade of CSE. As seen in Fig. 5, total amounts of glutathione excreted into bile was comparable irrespective of the PPG treatment, suggesting that this constituent plays little role in generation of the osmotic driving force, if any. Interestingly, the ratio between reduced and oxidized forms of glutathione (GSH/GSSG) was significantly elevated by the CSE blockade with PPG. Moreover, the PPG-induced elevation of GSH/GSSG in bile was further elevated with coperfusion with 30 μmol/L NaHS. As one might expect, the PPG pretreatment significantly caused a reduction of total glutathione presumably through inhibition of the transsulfuration pathway. The PPG-elicited decrease in hepatic glutathione contents was unchanged upon administration of NaHS, suggesting that the event is not mediated by endogenous H_2S. Among the three groups, >90% of glutathione was present as the reduced form (data not shown). These results suggest that suppression of CSE-derived H_2S accelerates biliary excretion of GSH, whereas its hepatic contents are reduced. Moreover, exogenous supplementation of the gas under the CSE blockade further increases its excretion into bile. Physiologic implications of this phenomenon will be mentioned later in the Discussion. Collectively, the present results suggest that H_2S endogenously generated by

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Possible Participation of Outer Mitochondrial Membrane Cytochrome B_5 in Steroidogenesis in Zona Glomerulosa of Rat Adrenal Cortex

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ABSTRACT

Outer mitochondrial membrane cytochrome b_5 (OMb) originally found in rat liver is an isoform of cytochrome b_5 (b_5) of the endoplasmic reticulum. In contrast to accumulated data on the physiological roles of b_5, functions of OMb have not been well characterized except for its involvement in regeneration of ascorbic acid [i.e., in a semidehydroascorbate reductase (SDAR) system]. By using highly specific antibodies against rat OMb, we found immunohistochemically that OMb in the rat adrenal gland was most abundant in the zona glomerulosa (zG) among the three cortical zones, and the expression level was enhanced on angiotensin II-stimulation. SDAR activity was found in zG and inhibited by anti-OMb antibody. Moreover, the increase in plasma aldosterone concentration under Na^+-deficiency was suppressed by limited ascorbic acid (Asc) availability in rat mutants unable to synthesize Asc, while plasma corticosterone concentration was not affected. These data suggest that OMb, present

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abundantly in zG, participates in aldosterone formation in zG of rat under angiotensin II-stimulation through regeneration of Asc.

Key Words: Cytochrome b_5; Zona glomerulosa; Steroidogenesis.

INTRODUCTION

Outer mitochondrial membrane cytochrome b_5 (termed "OMb") is an isoform of the well-known microsomal membrane cytochrome b_5 (termed b_5) and was originally found in the outer membrane of rat liver mitochondria (1). Both proteins are typical tail-anchored proteins, and their carboxyl-terminal portions with specific amino acids have been reported to target them to different organelles in a cell (2). The only function of OMb so far reported is participation in reduction of ascorbate free radical, semidehydroascorbate, to ascorbic acid (Asc), because the antibody against OMb, but not that against b_5, inhibited NADH-dependent semidehydroascorbate reductase (SDAR) activity (NADH: monodehydro-ascorbate oxidoreductase, EC 1.6.5.4) (3).

Recently, we have shown by using purified OMb and the specific antibody that only OMb, but not b_5, is present in rat testis and functions as a physiological modulator for testicular androgenesis (4). Involvement of OMb in the androgen synthesis in human tissues has been also reported (5).

It is well known that Asc is concentrated in the adrenal gland to very high levels compared with other organs (6) and that administration of ACTH induces a rapid decrease in Asc content followed by a slower decrease in cholesterol content in the adrenal gland (7). Besides a protective function of Asc as an antioxidant in steroidogenic enzyme systems (8), the function as an auxiliary electron donor for aldosterone-formation in the zona glomerulosa (zG) has been suggested (9,10). The present study was undertaken as the first step in studying roles of OMb in steroidogenesis of the adrenal cortex by using a specific antibody against OMb and employing an animal unable to synthesize Asc.

MATERIALS AND METHODS

Osteogenic disorder Shionogi (ODS) rats (male, body weight of about 200 g) were purchased from CLEA Japan, Inc. and used, unless otherwise stated. The ODS rat is a mutant Wistar rat genetically deficient in the ascorbic acid-synthesizing enzyme, L-gulonolactone oxidase (11). Since ODS rats grow normally as long as they are supplied with sufficient Asc, they were fed on ordinal lab chow with adequate Asc in the drinking water (0.1% Asc) until use. They were divided into four groups fed on Asc-deficient diet (Asc-deficient diet lab chow of CL-2 containing < 1 mg% of Asc, CLEA Japan Inc., Tokyo) with drinking water containing 0.1% Asc (group 1), rats fed on ordinal lab chow, 0.01% Asc (group 2), 0.009% Asc (group 3), and no Asc (group 4). In the other four groups (group 1′–4′), they were kept on Asc- and Na^+-deficient diet (CLEA Japan Inc., Tokyo) with various Asc-supplements corresponding to those in groups 1–4. The lab chow of CL-2 was autoclaved at 120°C for 7 min

before use to ensure the absence of Asc. The rats were kept for three weeks before sacrifice under the different conditions as described above.

Localization of OMb and b_5 on adrenal sections was performed using specific antibodies against rat OMb and b_5, respectively, as described before (4). Content of Asc in plasma was determined by essentially the same method as reported elsewhere (12) except that Asc was detected at 265 nm on high pressure liquid chromatography instead of an electrochemical detection system. Plasma concentrations of aldosterone, corticosterone and ACTH and renin activities were determined using commercially-available RIA kits. SDAR activities were measured according to the method of Ito et al. (3). Statistical significance was analyzed by unpaired Student's *t* test and analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Localization of OMb and SDAR Activities in the Adrenal Cortex

Figure 1 shows the localization of OMb and b_5 in the adrenal cortex from male Sprague–Dawley (SD) rat. OMb was immunohistochemically detected in the cortical cells but not in the medullary cells. In the cortex, OMb was more abundant in zG cells

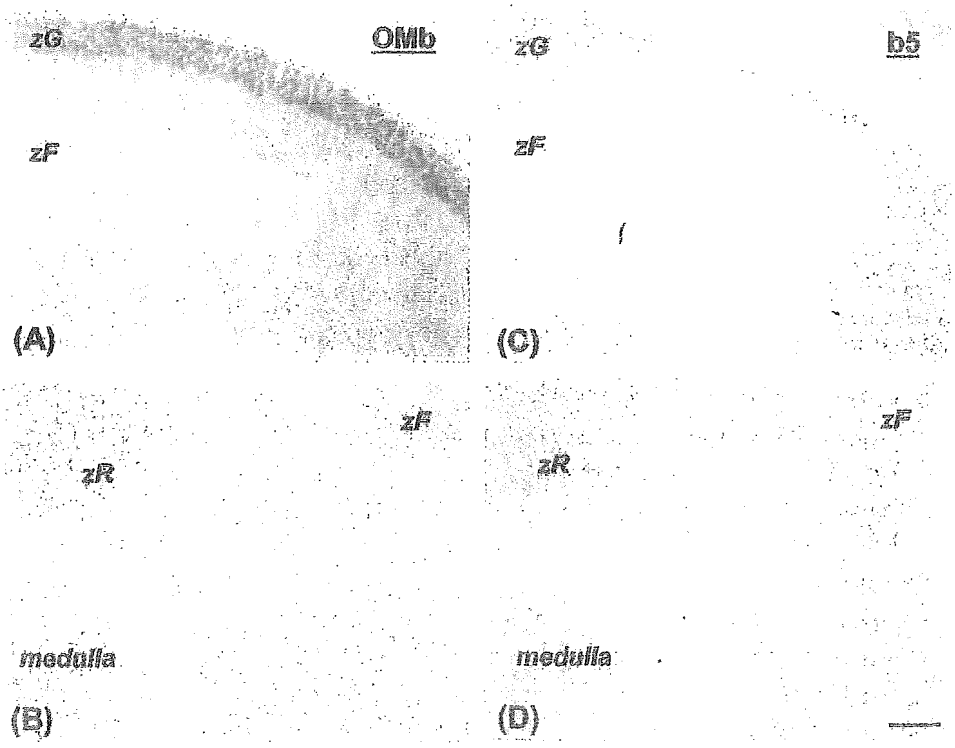

Figure 1. Localization of OMb and b_5 in the rat adrenal gland. Fresh-frozen adrenal sections (6 μm) from a male SD rat were treated with antirat OMb antibody (A and B) or antirat b_5 antibody (C and D). The immunoreactive proteins were visualized with 3,3′-diaminobenzidinetetrahydrochloride. Nuclei (light green) were poststained with methyl green. *Bars*, 100 μm.

Table 1. Semidehydroascorbate reductase (SDAR) activities in mitochondrial and microsomal fractions from zG and zFR-M of male SD rats.

Fractions	Activities*	Fractions	Activities*
zG Mit	30.26 ± 3.01	zFR-M Mit	29.98 ± 4.22
zG Mic	34.32 ± 5.32	zFR-M Mic	25.70 ± 1.53

*Activities: NADH oxidized nmol/mg protein/min (values: means ± SD, n = 3).

than in cells of other zones. On the other hand, b_5 was rather rich in the zonae fasciculata-reticularis (zFR) cells when compared with that in zG cells. In the medulla, b_5 was also undetectable. Thus, the majority of OMb and b_5 was zone-specifically localized in the adrenal cortex. The expression level of OMb in zG was increased upon angiotensin II stimulation under which the expression level of aldosterone synthesizing enzyme (P450aldo) was enhanced, and decreased on ACTH stimulation under which the expression level of P450aldo was suppressed.

SDAR activities in mitochondria (Mit) and microsomal (Mic) fractions from the capsular portion (mostly zG cells: zG) and the decapsular portion (mostly zFR and medullary cells: zFR-M) of the adrenal gland were then determined (Table 1). The activities were almost the same among fractions and inhibited by anti-OMb antibody by about 40%. These values were almost consistent with those reported by Nishino and Ito (13).

Possible Participation of OMb in Steroidogenesis in the Adrenal Cortex

Previous reports showed that the rate of steroidogenesis, especially aldosterone synthesis, in the adrenal cortex was enhanced by Asc (9,10). Therefore we investigated whether OMb is involved in Asc-supported steroidogenesis in the adrenal cortex. Since rats are capable of synthesizing Asc, we employed the ODS rat in which Asc-availability could be controlled.

As shown in Fig. 2, 0.009% of Asc concentration in drinking water (group 3) was found to be good enough to help ODS rats grow normally without scurvy. Under such conditions (group 3), the concentrations of Asc in plasma were less than 1/10 of those in animals receiving 0.1% Asc-supplement (group 1) (Fig. 3A). Concentrations of corticosterone (data not shown) and aldosterone (Fig. 3B, left) in plasma were almost the same among groups 1–4. When fed on Na-deficient diet (group 1′), renin activity and aldosterone concentration in plasma were increased by 3-fold (data not shown) and by 13-fold (Fig. 3B), respectively, in comparison with those of group 1. The degree of increment in aldosterone concentration, however, became reduced in parallel with the decrease in Asc-supplement (Fig. 3B, right): at 0.01% Asc-supplement (group 2′), plasma aldosterone concentration decreased to about 80%; at 0.009% Asc-supplement (group 3′) to about 60%; at no Asc-supplement (group 4′) to about 33% of the value at 0.1% Asc-supplement (group 1′). On the other hand, the concentration of corticosterone in plasma was unaffected by the degree of Asc-supplement and Na-deficiency.

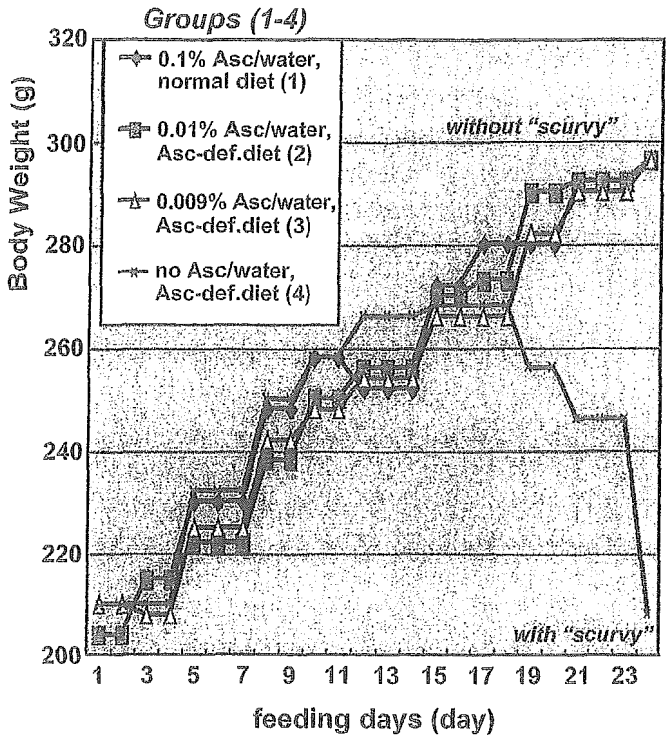

Figure 2. Changes in body weight of ODS rats under various Asc-supplementation in drinking water (groups 1–4). Means from 3 rats in individual group are plotted.

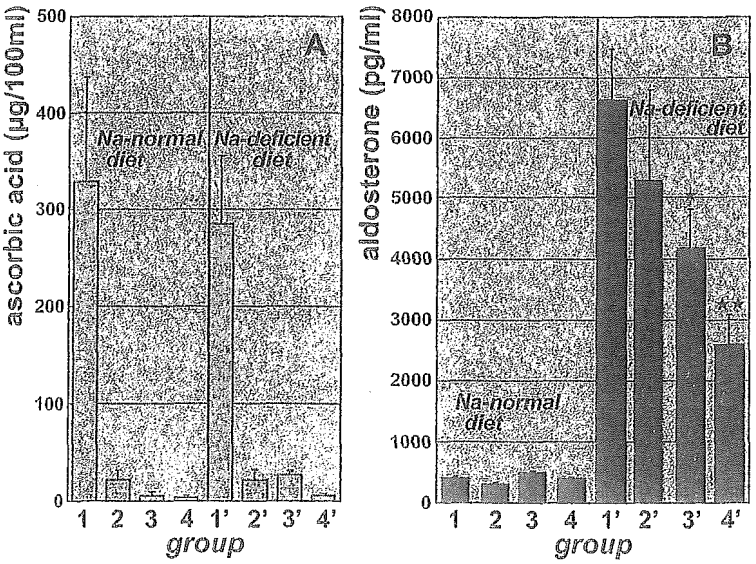

Figure 3. Concentrations of Asc (A) and aldosterone (B) in plasma from ODS rats of groups 1–4 and 1′–4′. Values: means ± SD, n = 3; $*p < 0.05$,$**p < 0.01$: significantly different from the value of group 1′ in (B).

Immunohistochemical studies showed that P450aldo was be expressed almost equally among adrenal cortices from the three Na^+-deficient groups (1′–3′).

In this study, OMb was shown for the first time to be present more abundantly in zG than in zFR of rat adrenal cortex. OMb seems to participate in aldosterone formation in zG of rat under angiotensin II-stimulation through regeneration of Asc. The detailed molecular mechanisms on these events await further investigation.

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Physiological Capacity of the Reticuloendothelial System for the Degradation of Hemoglobin Vesicles (Artificial Oxygen Carriers) after Massive Intravenous Doses by Daily Repeated Infusions for 14 Days

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ABSTRACT

A hemoglobin vesicle (HbV; diameter 252 ± 53 nm) or liposome-encapsulated Hb is an artificial oxygen carrier developed for use as a transfusion alternative, and its oxygen-transporting capacity has been well characterized, although critical physiological compartments for the Hb degradation after a massive infusion of HbV and the safety outcome remain unknown. In this study, we aimed to examine the compartments for its degradation by daily repeated infusions (DRI) of HbV, focusing on its influence on the reticuloendothelial system (RES). Male Wistar rats intravenously received the HbV suspension at 10 ml/kg/day for 14 consecutive days. The cumulative infusion volume (140 ml/kg) was equal to 2.5 times the whole blood volume (56 ml/kg). The animals tolerated the DRI well and survived, and body weights continuously increased. One day after DRI, hepatosplenomegaly occurred significantly through the accumulation of large amounts of HbV. Plasma clinical chemistry was overall normal, except for a transient elevation of lipid components derived from HbV. These symptoms subsided 14 days after DRI. Hemosiderin deposition and up-regulation of heme oxygenase-1 coincided in the liver and spleen but were not evident in the parenchyma of these organs. Furthermore, the plasma iron and bilirubin levels remained unchanged, suggesting that the heme-degrading capacity of the RES did not surpass the ability to eliminate bilirubin. In conclusion, phospholipid vesicles for the encapsulation of Hb would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of senescent RBCs, even at doses greater than putative clinical doses.

Phospholipid vesicles or liposomes have been extensively studied as a carrier of functional (macro)molecules for a drug delivery system, and some are now approved for clinical use as antifungal or anticancer therapies (Lian and Ho, 2001). Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb vesicles (HbV) or liposome-encapsulated Hb, have been developed as artificial oxygen carriers, and their sufficient ability to transport oxygen comparable with blood has been well clarified (Djordjevich et al., 1987; Chang et al., 1992; Izumi et al., 1997; Phillips et al., 1999; Sakai et al., 2004c). The advantages of an artificial oxygen carrier are the absence of blood-type antigens and transfusion-related transmission of infections, and stability during long-term storage. In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion that still has the potential of mismatching, the risk of infections secondary to the infusion of contaminated blood, and the problem of only a few weeks' storage life.

In a series of safety studies of HbV, it has been clarified that the cellular structure and the size of the HbV are advantageous for maintaining a steady blood circulation with-

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ABBREVIATIONS: HbV, Hb vesicle(s); Hct, hematocrit; RES, reticuloendothelial system; PEG, poly(ethylene glycol); RBC, red blood cell; DRI, daily repeated infusion(s); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadexyl-*N*-syccynyl-L-glutamate; MAP, mean arterial pressure; HR, heart rate; UA, uric acid; BUN, urea nitrogen; CRE, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; HO-1, heme oxygenase-1.

out vasoconstriction and hypertension (Sakai et al., 2000a) and that the surface modification of HbV with polyethylene glycol (PEG) is beneficial not only for a longer circulation time (Phillips et al., 1999) but also for suppression of intervesicular aggregation of HbV during preservation for years and in the plasma phase in the peripheral tissues after intravenous infusion (Sakai et al., 1998, 2000b). In our previous report on the histopathological analysis of rats receiving a bolus HbV infusion (20 ml/kg), the HbV particles are recognized as foreign materials and finally captured mainly by the reticuloendothelial system (RES, or mononuclear phagocytic system) in the spleen and liver, and they are promptly degraded (Sakai et al., 2001). These are outstanding characteristics in comparison with molecular Hb that shows a shorter circulation time because it is filtered through the kidneys when the Hb concentration exceeds the haptoglobin concentration and induces hemoglobinuria and eventually renal failure, and it extravasates across the fenestrated endothelium in the liver and induces excess heme catabolism in the hepatocytes and marked sinusoidal constriction (Goda et al., 1998, Kyokane et al., 2001). However, it is not clear whether the physiological capacity of the RES for the degradation and excretion of the components of HbV would be sufficient even after a massive infusion of HbV. The circulation half-life of HbV is within a few days, which is significantly shorter than that of red blood cells (RBCs), and it is anticipated that a massive infusion of HbV would burden the RES and result in abrupt heme degradation and iron overload having the potential to cause deleterious effects.

One of the safety studies of a new drug in the preclinical stage should be a massive dose by daily repeated infusions (DRI) in rodents and nonrodents for at least 14 days at three different dosages; a guideline decided by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. However, the documentation of the DRI studies to the public is scarce, especially in the research field of artificial oxygen carriers (Biro and Greenburg, 1999). In a clinical setting, the amount of an artificial oxygen carrier to be infused should be at least several hundred milliliters, which is significantly greater than the dose of conventional drugs; therefore, it is not clear whether a preclinical protocol for a conventional drug is appropriate for the safety evaluation of artificial oxygen carriers. On the other hand, there may be a need for a repetitive infusion of an oxygen carrier in a clinical situation, such as chronic anemia (Hamilton et al., 2001) or cancer therapy (Teicher et al., 1997). Based on these backgrounds, we tested the DRI of HbV into Wistar rats at one dose rate as a preliminary study to confirm the safety of HbV. Because the dose amount of phospholipid vesicles for use as an oxygen carrier is significantly greater than that used for conventional drugs, the influence of a massive infusion of HbV on the RES and the excretion of the components, especially after heme degradation, are of great concern.

Materials and Methods

Preparation of HbV Suspension. The test fluid, the HbV suspension, was prepared under sterile conditions as reported previously (Sakai et al., 2000b; Sou et al., 2000, 2003). Human Hb was purified from outdated, donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM pyridoxal 5′-phosphate (Aldrich Chemical Co., Milwaukee, WI) as an allosteric effector at a molar ratio of pyridoxal 5′-phosphate/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-*O*-dihexadecyl-*N*-succynyl-L-glutamate (DHSG) at a molar ratio of 5:5:1 (Nippon Fine Chemicals Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Co., Tokyo, Japan). Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DHSG/1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} was 5:5:1:0.033. HbVs were suspended in a physiological salt solution, sterilized using filters (pore size, 0.45 μm, Dismic; Toyo Roshi Co., Tokyo, Japan), and deoxygenated with bubbling N_2 for storage (Sakai et al., 2000b). The physicochemical parameters of the HbV are as follows: particle diameter, 252 ± 53 nm; [Hb], 9.5 g/dl; [metHb], 2.3%; [HbCO], <2%; [lipids], 5.3 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was measured by a modified *Limulus* amebocyte lysate gel-clotting analysis (Wako Pure Chemicals, Tokyo, Japan) and was less than 0.2 endotoxin unit/ml (Sakai et al., 2004a).

In our previous reports on resuscitation from hemorrhagic shock or extreme hemodilution, the HbV was suspended in a 5-g/dl albumin solution as a plasma expander to regulate the colloid osmotic pressure to 20 mm Hg (Sakai et al., 2004c). However, it is anticipated that the DRI of HbV suspended in albumin would result in enhanced hypervolemia. Because the main purpose of this DRI study was to clarify the safety of HbV and not albumin, HbV was simply suspended in a physiological saline solution.

Daily Repeated Infusion of HbV. All animal studies were approved by the Animal Subject Committee of the Keio University School of Medicine and performed according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 rev. 1985).

The experiments were carried out using 34 male Wistar rats (145 ± 4 g; Saitama Experimental Animals, Kawagoe, Japan). All the rats were housed in cages and provided with food and water ad libitum in a temperature-controlled room on a 12-h dark/light cycle. At first, the rats were anesthetized lightly with diethyl ether inhalation and then 1.5% sevoflurene (Maruishi Pharmaceutical Co., Osaka, Japan) using a vaporizer (model TK-4 Biomachinery; Kimura Medical, Tokyo, Japan) to immobilize them for every infusion. Every day for 14 days, the rats received HbV (n = 12) or saline (n = 12) via the tail vein using an indwelling needle (24-gauge; Nipro Co., Osaka, Japan) at a dose rate of 10 ml/kg with an injection rate of 1 ml/min. The total volume of the infused HbV into a rat for 14 days reached 140 ml/kg, which was equal to 2.5 times the actual blood volume of the rat (56 ml/kg). The infused total solid material (Hb and lipids) is calculated to be 20,689 mg/kg (1478 mg/kg/day × 14 days). The rats were weighed every day just before every infusion to calculate the amount of the infusion. After every infusion, the needle was immediately removed and the bleeding was stopped by applying pressure for a short time. The two groups (n = 12) were divided in half (n = 6 × 2), and six rats were sacrificed 1 day after the final 14th infusion. The remaining six rats were sacrificed at 14 days after the final infusion. Ten animals without the infusions were used to obtain control values.

Hematological Test. A hematological examination was performed at 1, 3, 7, 9, and 12 days during the DRI and at 1, 7, and 14 days after the final infusion. About 200 μl of blood was collected from a tail vein when an indwelling needle was inserted for HbV infusion. Seventy microliters was immediately diluted with 200 μl of citrate solution for a blood cell counter (Sysmex KX-21, Kobe, Japan), and the rest of the blood was inserted into a glass capillary (Terumo Co., Tokyo, Japan) for hematocrit (Hct) measurements. In this study, Hct indicates the volume of RBC and does not include the volume of HbV. The concentration of HbV in the plasma was measured by a cyanomethemoglobin method.

Hemodynamic and Blood Gas Parameters, Blood Glucose Level, and Urinalysis. One day or 2 weeks after the final infusion, the rats were anesthetized with 1.5% sevoflurene inhalation. A polyethylene tube (PE-50; Natsume Co., Tokyo, Japan) was inserted into the carotid artery for measurement of the mean arterial pressure (MAP), the heart rate (HR) by a recording system (Polygraph system 1000; Nippon Koden, Tokyo, Japan) and for withdrawing blood for various measurements. For the blood gas analysis, blood samples were collected in 70 IU/ml heparinized microtubes (125 μl, Clinitubes; Radiometer Nederland, Copenhagen, Denmark) and injected into a pH/blood gas analyzer (model ABL 555; Radiometer Nederland) for analyses of the arterial blood O_2 tension, arterial blood carbon dioxide tension, pH, base excess, and lactate. The blood glucose level was measured with a Medisafe Reader (GR-101; Terumo Co., Tokyo, Japan). Urinalysis was performed by dip-stick-testing (UA-L08M; Terumo Co.) as a qualitative measurement. A urine specimen of a rat was collected in a transparent plastic bag when the rat was lightly anesthetized with diethyl ether, and a test stick was dipped in the collected urine. In each item, the levels were judged by visual examination of the color identification after a specific time of exposure according to the instructions, in the order of protein (10 s), pH (10 s), occult blood (20 s), ketone body (20 s), urobilinogen (20 s), glucose (30 s), nitrite (30 s), and bilirubin (40 s).

Plasma Clinical Chemistry. A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma that was turbid and red/brown colored due to the presence of PEG-modified HbV particles, especially in the samples taken one day after DRI. The plasma was ultracentrifuged (50,000*g*; 20 min) to remove the HbV particles (Sakai et al., 2003). The obtained transparent plasma specimens were stored in a freezer at −80°C until the clinical chemistry tests (BML, Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, cholinesterase, leucine amino peptidase, creatine phosphokinase, amylase, lipase, aldosterone, total cholesterol, cholesterol ester, free cholesterol, HDL-cholesterol, β-lipoprotein, triglyceride, free fatty acid, phospholipids, total lipids, uric acid (UA), urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate, unsaturated iron binding capacity, and Fe^{3+}. All the analytical methods were described in our previous articles (Sakai et al., 2003, 2004b).

Blood Coagulation Test and Fibrinogen Concentration. For the blood coagulation test at 1 and 14 days after the final infusion of HbV or saline, 1.8 ml of the withdrawn blood was immediately mixed with 0.2 ml of 3.8% sodium citrate solution. The plasma fraction, obtained by gentle centrifugation, was analyzed for prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen (BML).

Histopathological Examination. The animals were finally laparotomized and sacrificed by acute bleeding from the abdominal aorta, and the liver, spleen, and kidney were resected for weight measurements and also all the other organs were obtained for a histopathological study. They were fixed in 10% buffered formalin (Wako Pure Chemicals) immediately after removal, and the paraffin sections were stained with hematoxylin & eosin, and Berlin blue.

Immunohistochemistry was performed to detect rat heme oxygenase-1 (HO-1) and human Hb from the injected HbV in the rat spleen and liver. Four-micrometer-thick paraffin sections were mounted on 3-aminopropyl triethoxysilane-coated glasses. The sections were treated with 0.03% H_2O_2 in methanol for 10 min at room temperature to block the endogenous peroxidase activity. For antigen retrieval, the sections were also treated with proteinase K (0.4 mg/ml; DakoCytomation California Inc., Carpinteria, CA) for 10 min at room temperature. After blocking the nonspecific binding with 5% normal goat serum, they were incubated with mouse monoclonal antibody against rat HO-1 (20 μg/ml; GTS-3, TaKaRa, Tokyo, Japan) at 4°C overnight. They were then incubated for 30 min at room temperature with goat antibodies against mouse immunoglobulins conjugated to the amino acid polymer [no dilution; Histofine Simple Stain MAX-PO(M), Nichirei Co., Tokyo, Japan]. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (0.2 mg/ml; Dojindo Laboratories, Kumamoto, Japan) in 0.05 M Tris-HCl, pH 7.4, containing 0.003% hydrogen peroxide. Subsequently, the sections were treated with 5% normal swine serum for 30 min at room temperature and reacted with rabbit polyclonal antibodies against human Hb (1:500 dilution; DakoCytomation A/S, Glostrup, Denmark) for 60 min at room temperature. They were further incubated with alkaline phosphatase-conjugated swine antibodies against rabbit immunoglobulins (1:100 dilution; DakoCytomation A/S). Color development was performed using a New Fuchsin Substrate kit (Nichirei Co.), and the sections were counterstained with hematoxylin.

Data Analysis. Differences between the control and the treatment group were analyzed using a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered statistically significant if $p < 0.05$. All the data are shown as mean ± S.D. For the results of the plasma clinical chemistry, the allowance of twice the standard deviation (2× S.D.) of the baseline values is indicated in the figures in considering the variable nature of these parameters.

Results

Body Weight. The body weight of rats in the HbV group (baseline, 144 ± 3 g) showed a monotonous increase during the 14 days of the DRI period and reached 195 ± 12 g (Fig. 1); however, this was slightly but significantly suppressed ($p < 0.05$) in comparison with the control saline group (220 ± 13 g). The body weight in the HbV group increased to 265 ± 14 g at 14 days after DRI. No significant difference was noted in the body weight compared with that of the saline control group (280 ± 22 g).

Hematological Changes and Concentration of HbV in Blood. The Hct of the HbV group (baseline, 41.7 ± 2.1%) tended to decrease to 37.5 ± 0.9% 1 day after DRI, which was lower than that of the saline group (44.7 ± 2.0%) (Fig. 2). However, after 14 days, the Hct of the HbV group increased to 45.1 ± 1.9%, which was comparable with that of the saline group (47.8 ± 2.7%). The numbers of white blood cells and platelets were comparable with those of the saline control group throughout the observation period.

The concentration of HbV immediately after every infusion was estimated from the volumes of the whole blood (56 ml/kg) and the infused volume of HbV (10 ml/kg) and was plotted

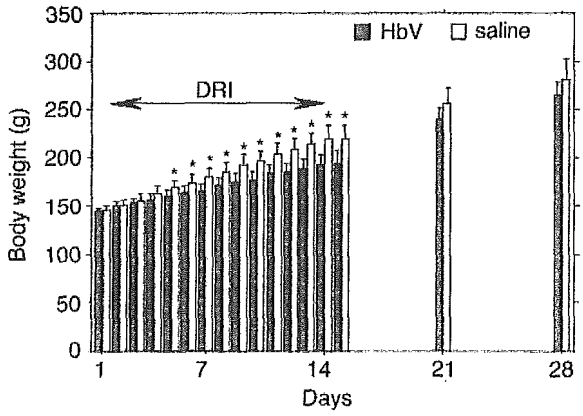

Fig. 1. Time course of the gain in body weight during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. Both groups showed monotonous increases; however, after the 5th day, a significant difference was observed. Seven days after the final infusion, there were no significant differences between the two groups. The values are mean ± S.D. *, significantly different between the groups ($p < 0.05$).

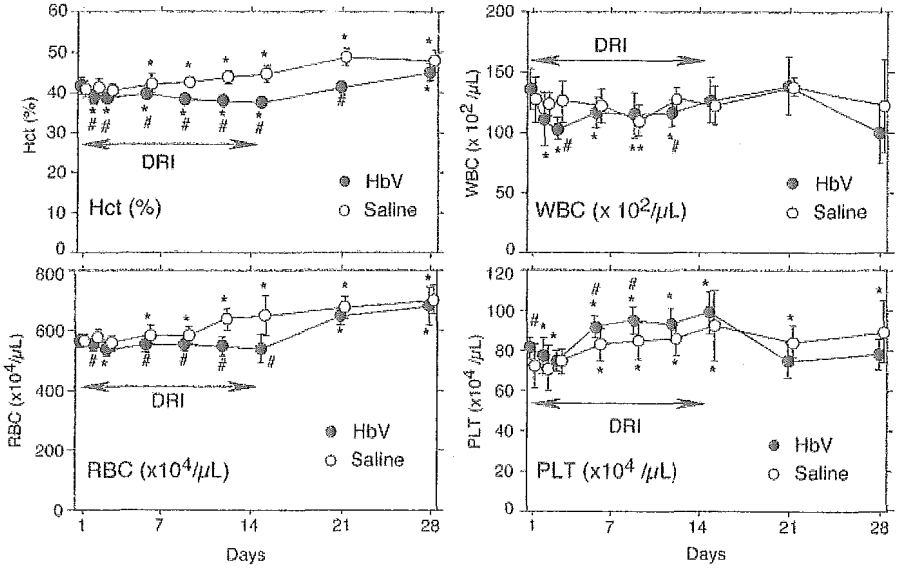

Fig. 2. Time course of Hct and blood cell counts during and after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. #, significantly different between the groups ($p < 0.05$). *, significantly different versus the baseline values ($p < 0.05$).

with open circles, and the measured concentration of HbV just before every infusion was plotted with solid circles (Fig. 3). The concentration of HbV just after the first infusion was estimated to be about 3 g/dl, and 1 day later, it decreased to 1.3 g/dl, with a half-life of about 22 h. The half-life of the second infusion seemed to become shorter. The DRI resulted in the accumulation of HbV in the plasma and it increased to 5.2 g/dl 1 day after the 14th infusion. However, 1 week after the final infusion, no HbV was confirmed in the plasma, indicating that all of the HbV was captured by the RES.

Coagulation Test. A coagulation test indicated that the HbV group showed a slight prolongation in APTT from 24.3 ± 1.3 s at the baseline to 36.0 ± 11.8 s 1 day after the DRI but changed to 40.3 ± 20.3 s after 14 days with marked individual variations. On the other hand, there was no noticeable change in the PT for the HbV group (Fig. 4). The fibrinogen concentration (baseline, 223 ± 12 mg/dl) significantly increased for all groups (HbV group, 1 day after DRI,

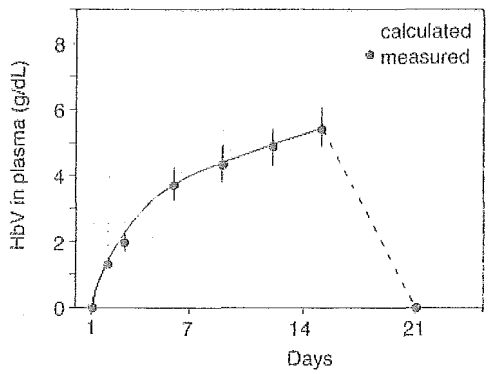

Fig. 3. Time course of the HbV concentration in the plasma phase during and after DRI of HbV for 14 days at a dose rate of 10 ml/kg/day. It is assumed that immediately after the first infusion, the concentration should be around 3 g/dl, and 1 day later, it decreased to about 1.2 g/dl. Immediately after the second infusion, the concentration should be about 4.3 g/dl and decreased to 2 g/dl 1 day later. The half-life of the HbV apparently decreases with multiple infusions. The concentration tended to reach a plateau. The final HbV concentration reached 5.3 g/dl, and this completely disappeared 7 days after DRI.

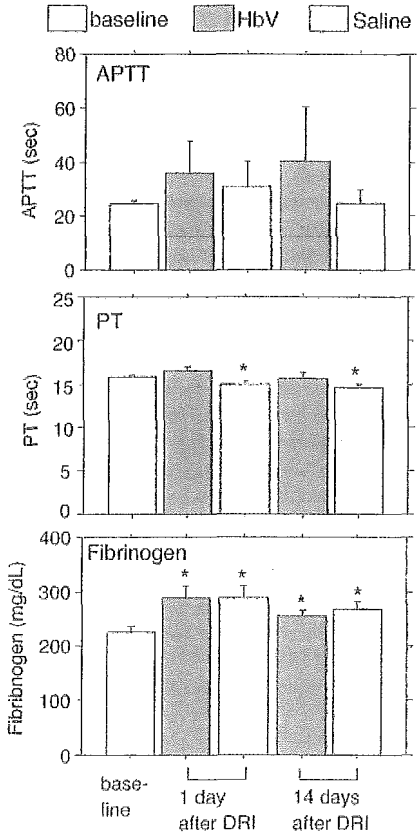

Fig. 4. Parameters for blood coagulation, APTT and PT, and fibrinogen concentration after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

289 ± 22 mg/dl; 14 days after DRI, 255 ± 11 mg/dl), probably due to the stress of infusion and influence on the liver function.

Blood Pressure, Heart Rate, and Blood Gas Parameters. Table 1 summarizes the blood gas parameters, blood glucose level, MAP, and HR 1 day after the DRI of HbV. There is no abnormal value except for an increase in MAP of

TABLE 1

Blood gas parameters, blood glucose level, MAP, and HR 1 and 14 days after DRI of HbV

Baseline values are also listed. The values are mean ± SD.

Parameters	1 Day after DRI	14 Days after DRI	Baseline
pH	7.44 ± 0.03	7.43 ± 0.03	7.48 ± 0.30
$PaCO_2$ (torr)	41 ± 3	44 ± 4	37 ± 4
PaO_2 (torr)	76 ± 4	75 ± 7	82 ± 7
Lactate (mM)	0.93 ± 0.12	0.88 ± 0.38	1.59 ± 0.45
Base excess (mM)	3.1 ± 1.3	4.0 ± 0.8	4.5 ± 1.3
Glucose (mg/dl)	147 ± 24	127 ± 9	146 ± 10
MAP (mm Hg)	125 ± 4*	111 ± 12	101 ± 8
HR (beats/min)	419 ± 10	402 ± 33	404 ± 42

* Significantly different versus the baseline group ($p < 0.05$).

the HbV group 1 day after DRI (125 ± 4 mm Hg) in comparison with the baseline value (101 ± 8 mm Hg).

Urinalysis. The color of the urine was normal for all groups, and there was no sign of hemoglobinurea. Table 2 summarizes the results of the urinalysis. The HbV group showed a slight increase in the protein concentration. There were no significant signs of any organ damage. Urobilinogen and bilirubin were within the normal range in spite of the large amount of HbV infusion.

Organ Weights. The liver and spleen are thought to be the main organs that trap and degrade HbV. As shown in Fig. 5, significant splenomegaly and hepatomegaly were confirmed 1 day after DRI. The percentage of spleen weight relative to the body weight increased from 0.33 ± 0.04% at the baseline to 0.94 ± 0.07 1 day after DRI, about 2.9 times the baseline value. This returned to 0.41 ± 0.03% after 14 days. The percentage of liver weight relative to the body weight increased from 4.81 ± 0.15% at the baseline to 5.83 ± 0.37% 1 day after DRI; and it returned to 4.33 ± 0.20%, comparable with the baseline after 14 days. The color of the liver was darkened just after DRI, however, it returned to its normal color 14 days after DRI. The kidney weight did not show any significant increase but tended to show a slight decrease for all groups.

Plasma Clinical Chemistry. A significant amount of the HbV particles was present in the plasma one day after DRI. However, they could be easily removed from the plasma by ultracentrifugation (50,000*g*; 20 min) (Sakai et al., 2003), and we could avoid any interference effect of HbV in the colorimetric and turbidimetric analyses in the plasma clinical chemistry. The parameters affecting the liver function (total

TABLE 2

The results of urinalysis for the HbV and saline control groups 1 and 14 days after DRI ($n = 6$)

The numbers indicate the counts of rats. Interpretation of judgment for the reading levels: protein (mg/dl), − (negative), ± (15), + (30), ++ (100), +++ (250), and ++++ (1000); occult blood (counts/μl), − (negative), + (10), ++ (50), and +++ (250); ketone body (mg/dl), − (negative), + (10), ++ (50), and +++ (100); urobilinogen (mg/dl), − (negative), ± (0.5), + (2), ++ (4), and +++ (8); glucose (mg/dl), − (negative), ± (50), + (150), ++ (500), and +++ (2000); nitrite, − (negative), and + (0.03–0.2 mg/dl); and bilirubin (mg/dl), − (negative), + (0.5), ++ (1), and +++ (2.5).

Parameters	Levels	1 Day after DRI		14 Days after DRI		Baseline
		HbV	Saline	HbV	Saline[a]	
Protein	−	0	1	2	0	2
	±	4	5	1	2	4
	+	2	0	2	2	0
	++	0	0	1	1	0
	+++	0	0	0	0	0
	++++	0	0	0	0	0
pH	5	0	2	0	0	0
	6	6	2	2	2	0
	7	0	1	4	2	5
	8	0	1	0	1	1
	9	0	0	0	0	0
Occult blood	−	3	2	4	3	4
	+	3	4	2	1	1
	++	0	0	0	1	1
	+++	0	0	0	0	0
Ketone body	−	0	0	0	0	0
	+	6	6	5	5	6
	++	0	0	1	0	0
	+++	0	0	0	0	0
Urobilinogen	−	0	0	0	0	0
	±	6	6	6	5	6
	+	0	0	0	0	0
	++	0	0	0	0	0
Glucose	−	6	6	6	5	6
	±	0	0	0	0	0
	+	0	0	0	0	0
	++	0	0	0	0	0
Nitrite	−	6	6	6	5	6
	+	0	0	0	0	0
Bilirubin	−	6	6	6	5	5
	±	0	0	0	0	1
	+	0	0	0	0	0
	++	0	0	0	0	0
	+++	0	0	0	0	0

[a] $n = 5$.

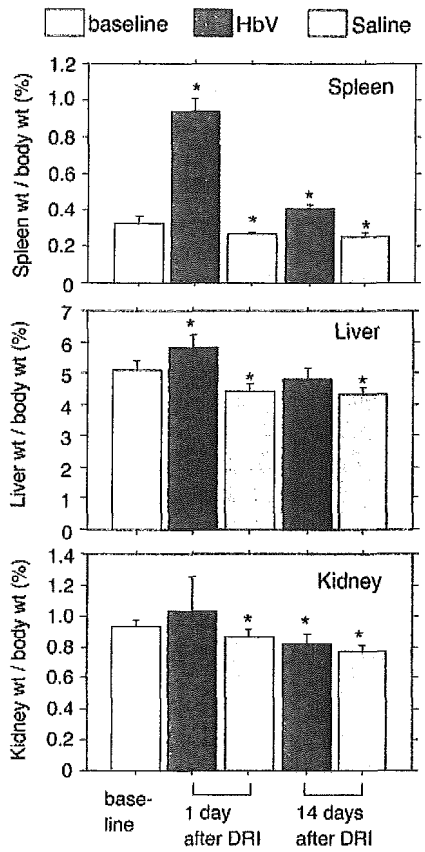

Fig. 5. Changes in organ weights (spleen, liver, and kidney) after DRI of HbV and saline for 14 days at a dose rate of 10 ml/kg/day. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$).

protein, albumin, aspartate aminotransferase, alanine aminotransferase, leucine amino peptidase, alkaline phosphatase, γ-glutamyltransferase, and cholinesterase) did not show any noteworthy changes in the HbV group (Fig. 6).

The parameters affecting the function of the kidneys (CRE, uric acid, and BUN) varied within the normal ranges. The parameter reflecting the heart and skeletal muscle, creatine phosphokinase, did not show any noticeable change. Aldosterone, released from adrenal gland to regulate ionic balance, did not show noticeable change. A significant change was observed for the parameters of pancreatic function. In the HbV group, the lipase activity (baseline, 8.5 ± 1.4 U/l) increased to 48.5 ± 16.8 U/l 1 day after DRI and tended to decrease to 33.2 ± 29.4 U/l after 14 days. On the contrary, amylase activity (baseline, 1613 ± 74 U/l) did not show an increase but a slight decrease to 1455 ± 28 U/l 1 day after DRI and returned to 1546 ± 77 U/l after 14 days.

The concentrations of the cholesterol components (total and free cholesterols, and cholesterol ester) and lipids (β-lipoprotein, total lipids, and phospholipids) significantly increased 1 day after the final infusion (Fig. 7). For example, total cholesterol (baseline, 72.6 ± 7.5 mg/dl) increased to 182.2 ± 22.6 mg/dl after DRI. However, they returned to the original values 14 days after DRI. These increases should indicate that cholesterol and phospholipid (probably DPPC) are released from the RES after entrapping the HbV particles. Bilirubin and ferric iron, which should be released from the Hb decomposition, were minimal. Unsaturated iron binding capacity did not show noticeable changes. The electrolyte concentrations varied within the normal range.

Histopathological Examination. Histopathological examination 1 day after DRI showed a significant amount of HbV accumulated in the red pulp zone in the spleen, and very few RBCs were seen (Fig. 8a). In the liver, the presence of Kupffer cells that captured a large amount of HbV was seen (Fig. 8b). In the kidneys, the mesangial cells in the renal glomerulus seemed to entrap HbV (Fig. 8c). These organs and the adrenal gland were slightly stained with Berlin blue (data not shown), indicating that the decomposition of heme should have already started. No morphological change was noted in the myocardium; however, some slightly stained particles were observed (Fig. 8d). The pancreas (Fig. 8e), lungs (Fig. 8f), intestine, stomach, brain, thymus, testis, and skin did not show significant abnormalities.

Fourteen days after DRI, the images of the accumulated HbV almost disappeared in all organs. However, there were materials that were moderately stained with Berlin blue in the red pulp zone of the spleen (Fig. 9a), liver (Fig. 9b), bone marrow (Fig. 9c), and slightly in the kidney (Fig. 9d) and adrenal gland (Fig. 9e)

Immunohistochemical analysis of the liver and the spleen clarified the presence of human Hb in HbV as pink-colored areas that were stained with anti-human Hb-antibody 1 day after DRI (Fig. 10, a and b). In the spleen, the presence of HO-1 was confirmed as brown-colored stains in the cytoplasm of the macrophages in the red pulp zone both at 1 and 14 days after DRI (Fig. 10, a and c). In the liver, the presence of HO-1 was confirmed in the Kupffer cells only at 14 days after DRI (Fig. 10d). No HO-1 was confirmed in the parenchyma of these organs.

Discussion

Our primary finding is that all the rats tolerated the DRI of HbV well for 14 days with no deteriorative signs in organ functions, due to the preferable effect of Hb encapsulation in phospholipid vesicles that minimizes the toxicity of molecular Hbs and delivers them to the RES as a physiological compartment for degradation and detoxification of foreign materials. The RES had sufficient capacity for the degradation of HbV, even though the total infused volume reached 140 ml/kg, which was equal to 2.5 times the actual blood volume of the rat (56 ml/kg) and was significantly larger than the dose of multiple infusions of liposomes for antifungal and antitumor targeting (Fielding et al., 1999; Charrois and Allen, 2003).

The body weight of the HbV group monotonously increased, whereas the rate was slightly slower than that of the saline control group. It is speculated that the infusion of HbV, which could not be excreted easily in the urine and remain in circulation, could 1) disturb physiological functions and suppress the growth of the animals, 2) put the animals under stress and reduce their appetite, or 3) tend to accelerate the catabolism. In spite of such a condition, the components of HbV could be used as a part of the cellular components for the growth of rats. One and 2 weeks after DRI, there was no significant difference in the body weight between the groups.

The numbers of RBCs, whole blood cells, and platelets showed moderate changes, even though there were some

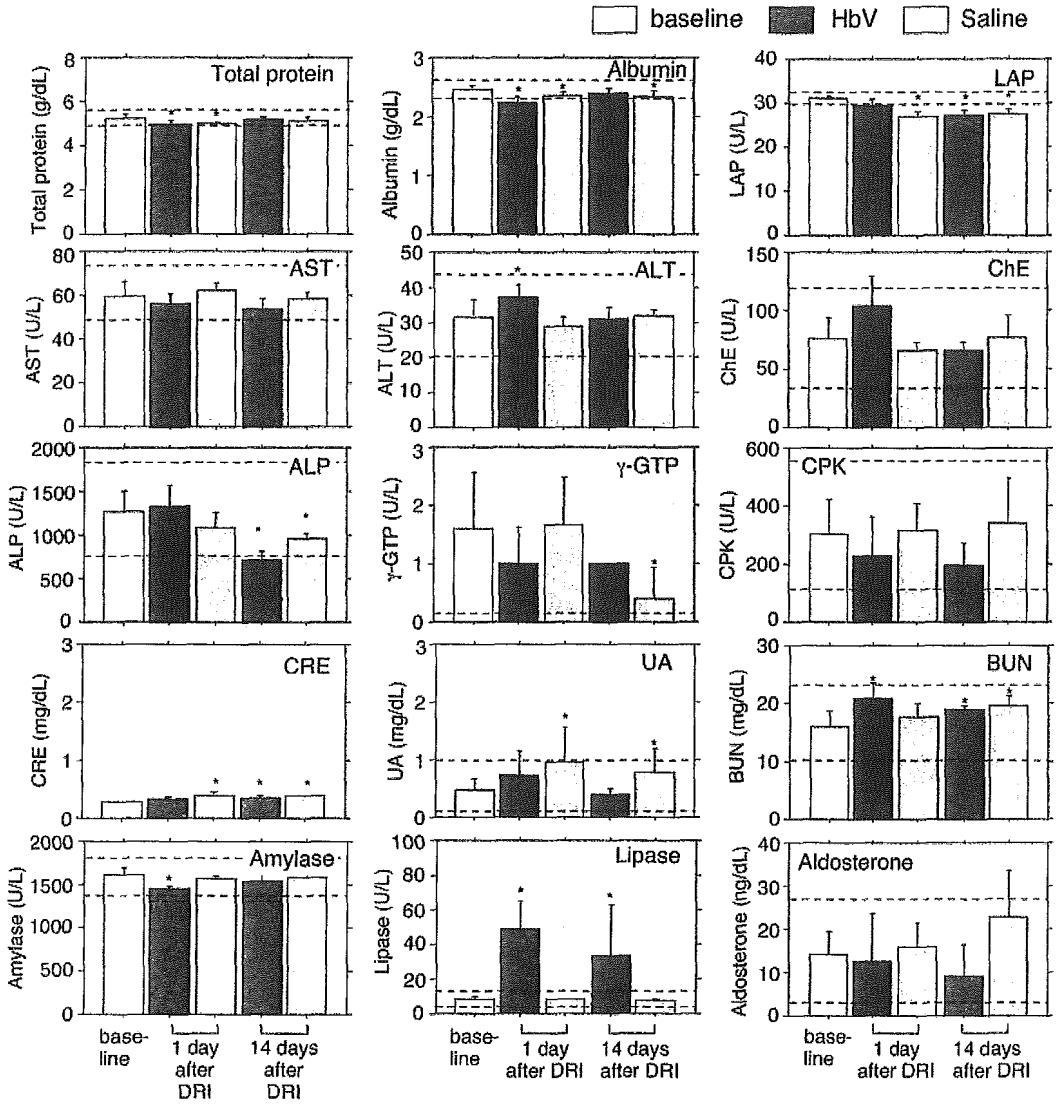

Fig. 6. Plasma clinical chemistry tests reflecting the organ functions such as liver, pancreas, and kidneys and the metabolism of Hb after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group ($p < 0.05$). The dotted lines indicate the levels of 2× S.D. LAP, leucin amino peptidase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ChE, cholinesterase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase; CPK, creatine phosphokinase; CRE, creatinine; UA, uric acid; BUN, urea nitrogen.

significant differences between the HbV and the saline groups. Hct and RBC counts decreased significantly for the HbV group, probably due to the dilution of blood by hypervolemia, or suppression of erythropoiesis (release of erythropoietin) because the renal cortex would be exposed to the increased oxygen content in the blood during DRI of HbV as oxygen carriers. The slight hypertension 1 day after DRI would be related to the blood hyperviscosity or hypervolemia due to the presence of HbV. However, the Hct and RBC counts returned to levels similar to those of the saline group 14 days after DRI. The time course of the HbV concentration in plasma indicates that the rate of HbV clearance gradually increased and the concentration reached a plateau, probably due to the nonspecific phagocytic activation of the RES that was clarified previously by a carbon clearance measurement (Sakai et al., 2001). The accelerated liposome clearance of the second infusion was well characterized (Claassen et al., 1988; Laverman et al., 2001); however, its mechanism, antibody formation or complement activation is controversial (Dams et al., 2000; Ishida et al., 2003).

In our previous report, the bolus HbV infusion (20 ml/kg) resulted in significant splenomegaly (about 100% increase) and hepatomegaly (13%) (Sakai et al., 2004b). In the present DRI study, splenomegaly was enhanced (190%), whereas hepatomegaly was similar (14%), indicating that the spleen had a larger capacity for HbV clearance. A large amount of HbV accumulated in the red pulp zone of the spleen and in Kupffer cells of the liver; however, 14 days later it disappeared and the splenohepatomegaly completely subsided. The spleen and the liver showed significant hemosiderin deposition; however, the enzyme concentrations that reflect the liver function did not show any abnormal values.

One day after DRI, the mesangial cells in the renal glomerulus seemed to entrap HbV in their intracellular spaces, and the same portion was stained with Berlin blue 1 and 14 days after DRI. In our previous report on the bolus HbV infusion, there was no abnormality in the kidneys (Sakai et al., 2004b). According to Rudolph et al. (1995), liposome-encapsulated Hb without PEG-modification aggregated in the plasma and showed a slight accumulation in the kidneys. Even though our PEG-modified HbV does not induce intervesicular aggregation, HbV would tend to be aggregated during the longer circulation time due to the DRI. No abnormal value was noted for UA, BUN, and CRE, although urinalysis showed a slight increase in protein levels.

Lipase activity, but not that of amylase, significantly in-

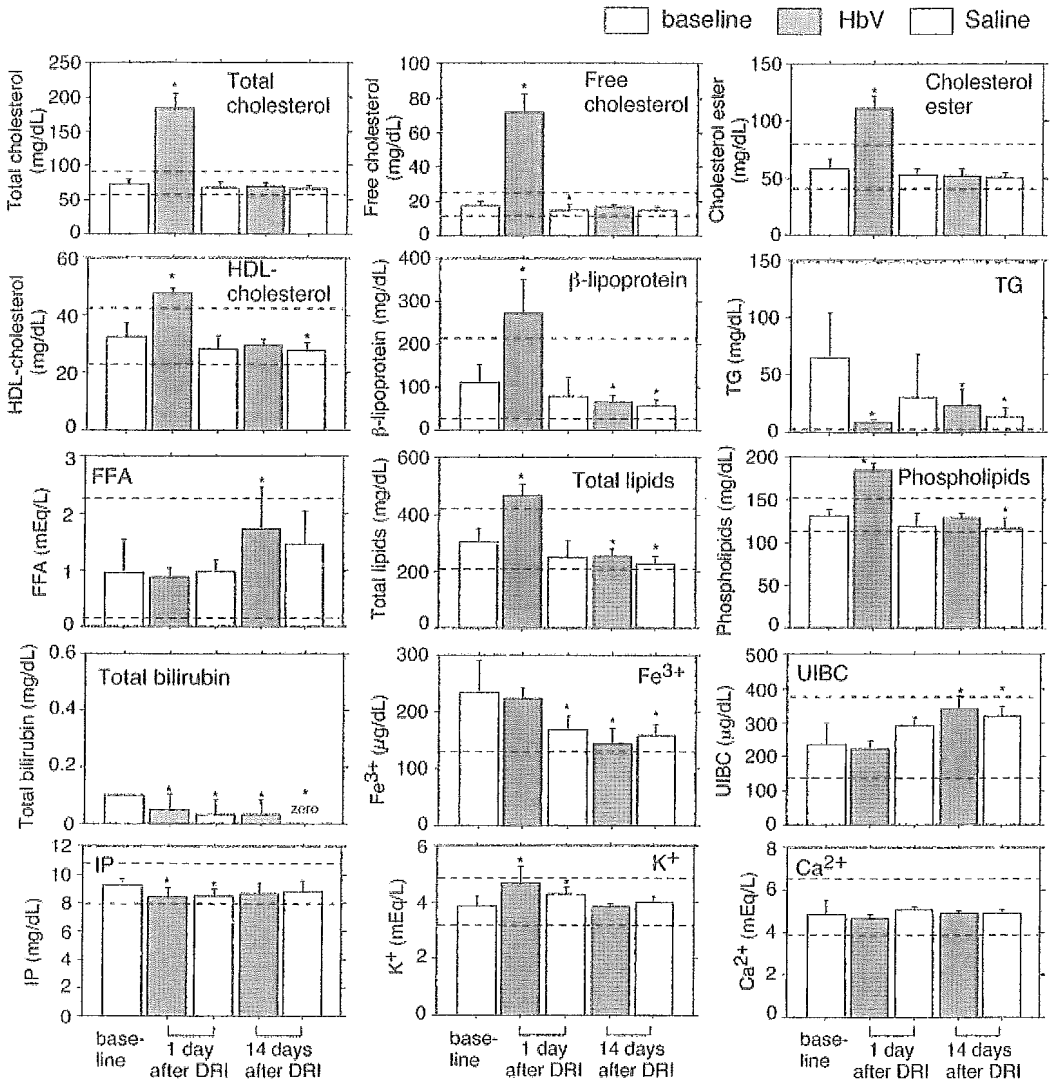

Fig. 7. Plasma clinical chemistry tests reflecting the metabolism of lipids and Hb and electrolytes 1 or 14 days after DRI of HbV or saline. The values are mean ± S.D. *, significantly different versus the baseline group. The dotted lines indicate the levels of 2× S.D. TG, triglyceride; FFA, free fatty acid; UIBC, unsaturated iron-binding capacity; IP, inorganic phosphate.

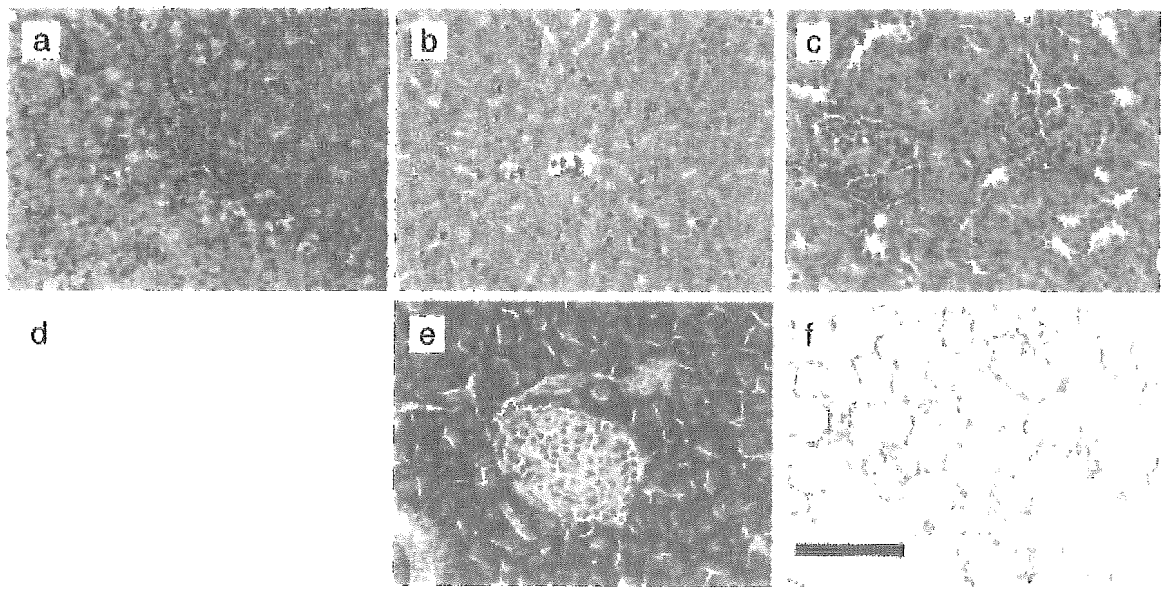

Fig. 8. Histology of spleen (a), liver (b), kidneys (c), heart (d), pancreas (e), and lungs (f) 1 day after DRI of HbV. A significant amount of HbV was accumulated in the red pulp zone of the spleen. The invasion of a significant number of Kupffer cells with HbV was seen in the liver. In the kidneys, the mesangial cells in the renal glomeruli seemed to entrap HbV. The myocardium showed slight staining with Berlin blue. No significant pathological changes are noted in the pancreas and lungs. Scale bar, 100 μm. Hematoxylin and eosin stains (a, b, c, e, and f) and Berlin blue stain (d).

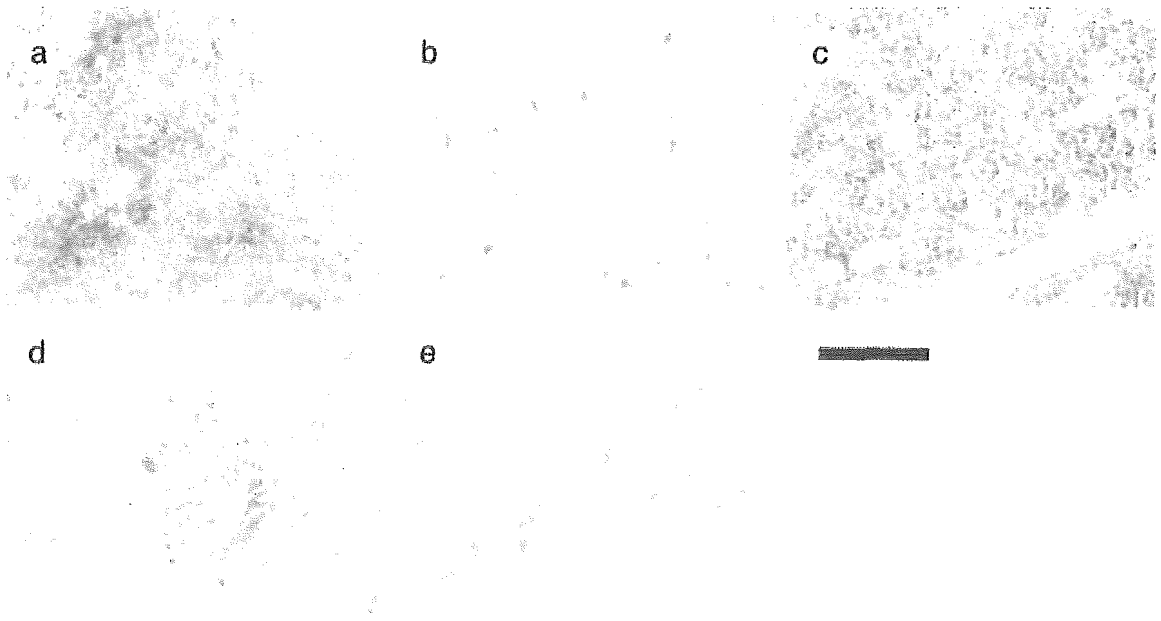

Fig. 9. Histology of spleen (a), liver (b), bone marrow (c), kidneys (d), and adrenal gland (e) 14 days after DRI. Berlin blue staining was performed to examine the presence of hemosiderin. Scale bar, 100 μm.

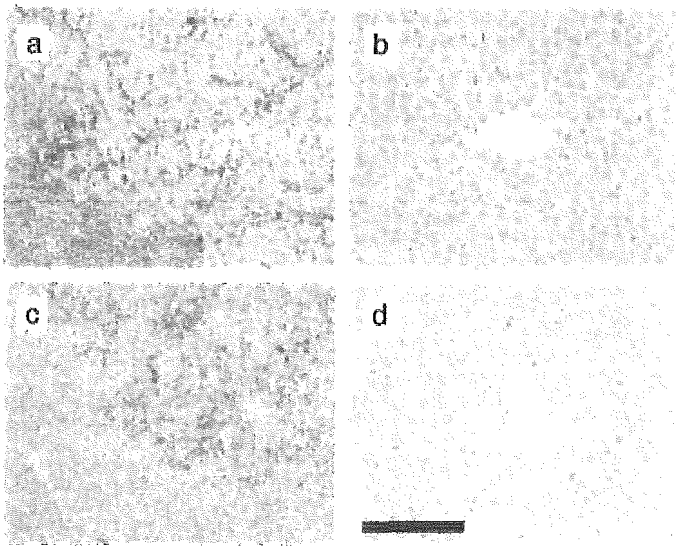

Fig. 10. Double immunohistochemical staining for HO-1 and human Hb in HbV in the rat spleen (a and c) and liver (b and d), 1 (a and b) and 14 days (c and d) after DRI of HbV. The tissues were stained with anti-rat HO-1 monoclonal antibody (GTS-3). The brown-colored portions (a, c, and d) indicate the presence of HO-1, and the pink or gray-beige areas (a and b) indicate the presence of a large amount of HbV. Scale bar, 100 μm.

creased in the HbV group, whereas there was no histopathological abnormality in the pancreas. A similar tendency was observed after the bolus HbV infusion (20 ml/kg) (Sakai et al., 2004b). This level of increment was significantly smaller than the value for the Wistar rats with acute necrotizing pancreatitis that increased the lipase activity from 10 to 475 to 5430 IU/l (Hofbauer et al., 1996). One possible reason for the moderate and specific increase in lipase activity would be related to the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes (Stuecklin-Utsch et al., 2002), because pancreatic lipase hydrolyzes not only triglyceride but also phosphatidylcholine (Rowland and Woodley, 1980). However, the mechanism is not clear, and the pancreatic function should be carefully monitored in the ongoing safety studies.

The plasma lipid components significantly increased after the DRI of HbV. They should be derived from HbV because it contains a large amount of cholesterol and DPPC, and they would be liberated after the HbV particles are captured and degraded in the RES. It is reported that once liposome is captured in the Kupffer cells, the diacylphosphatidylcholine is metabolized and is reused as a cell membrane component or excreted in the bile (Dijkstra et al., 1985; Verkade et al., 1991). Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV (diameter, 250 nm) cannot diffuse across the fenestrated endothelium into the space of Disse (Goda et al., 1998). Cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al., 1986). Judging from the results showing that the increases in the plasma lipid components were transient, the lipid components of HbV would gradually be redistributed, metabolized, and excreted in the same manner within 14 days after DRI. However, the details have to be confirmed by the biodistribution of the radiolabeled components.

In spite of the massive HbV infusions, the plasma bilirubin and iron levels did not increase. Urinalysis also showed no increase in the urobilinogen and bilirubin. The anti-human Hb antibody staining detected temporal distributions of HbV in the spleen and liver. The excess amount of heme from Hb in HbV should be metabolized by the inducible form of HO-1 in the spleen macrophages and the liver Kupffer cells, as shown in Fig. 10 (Braggins et al., 1986; Goda et al., 1998). Bilirubin should be excreted in the bile as a normal physiological pathway even during the massive doses of HbV. No increase in the plasma bilirubin level indicated that there was no obstruction or stasis of bile in the biliary tree and that the heme-degrading capacity of the RES did not surpass the ability to eliminate

bilirubin. Berlin blue staining revealed the presence of hemosiderin in the liver, spleen, kidneys, adrenal gland, and bone marrow 14 days after DRI and also in the myocardium 1 day after DRI. Both ferritin and hemosiderein store and release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert (O'Connell et al., 1989). Multiple blood transfusions often induce hemosiderosis in many organs. Accordingly, Hb encapsulation in the phospholipid vesicles would guarantee the smooth metabolic route of HbV that is similar to the well characterized metabolic route of senescent RBCs in the liver Kupffer cells and spleen macrophages (Bennett and Kay, 1981; Hirano et al., 2001). This would be a great advantage over molecular Hb that incurs not only filtration across the fenestrated endothelium of the glomerular capillary in the kidneys resulting in shorter circulation time and renal failure but also extravasation from the sinusoidal caliber in the liver, causing cancellation of the CO-mediated fail-safe mechanism for conserving sinusoidal patency and bile formation (Kyokane et al., 2001).

In conclusion, all the rats tolerated the DRI of HbV with no deteriorative signs of the organ functions. The phospholipid vesicles for Hb encapsulation would be beneficial for heme detoxification through their preferential delivery to the RES, a physiological compartment for degradation of not only foreign materials but also the senescent RBCs. However, it has to be considered that in humans the circulation time of HbV and its degradation rate in the RES would be different compared with those in rats, because the circulation time of stealth liposomes and the life span of RBCs are different between rodents and humans (Landaw, 1988; Gabizon et al., 2003). A shock condition may also influence on the RES function.

Our results would provide important information not only for the ongoing safety studies of HbV but also for the overall research on liposomal drugs, because this study is the first attempt to infuse repetitively such a large amount of phospholipid vesicles.

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