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Absence of Anaphylactic Reactions to Injection of Hemoglobin Vesicles (Artificial Red Cells) to Rodents

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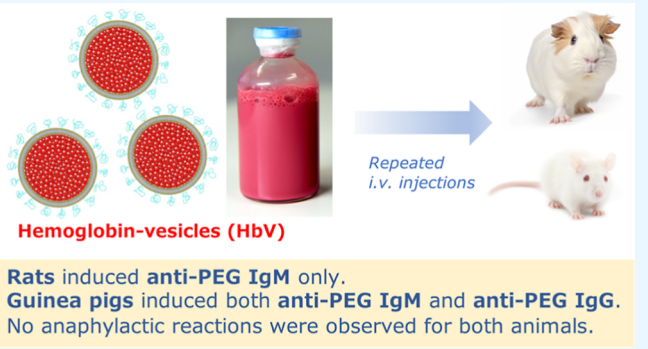
ABSTRACT: The safety and efficacy of hemoglobin vesicles (HbVs) as artificial oxygen carriers encapsulating a purified and concentrated Hb solution in liposomes have been studied extensively. The HbV surface, modified with PEG by incorporating a PEG-conjugated phospholipid, is beneficial for storage and biocompatibility. However, it might be possible that interaction of PEG and the pre-existing anti-PEG antibody in the bloodstream causes acute adverse reaction. This study used two sets of experiments with rats and guinea pigs to ascertain whether the anti-PEG antibody generated by the PEG-modified HbV injection can induce anaphylactic reactions. SD rats received repeated intravenous injection of HbV at a dose rate of 16 or 32 mL/kg three times. Not anti-PEG IgG but anti-PEG IgM was detected. Nevertheless, no anaphylactic reaction occurred. Guinea pigs were used to study the presence of active systemic anaphylaxis further after injections of the PEG-modified liposomes used for HbV. The animals were sensitized by three repeated subcutaneous injections of PEG-modified liposomes (PEG−liposome) along with adjuvant at 1 week intervals. For comparison, unmodified liposomes (liposome) and 10 times excessively PEG-modified liposomes with ionizable lipid (10PEG−DODAP−liposome) were used. Inclusion of PEG modification induced not only anti-PEG IgM but also anti-PEG IgG. Three weeks after the final injection, intravenous injection of both PEG−liposome and liposome (1 mL/kg) induced no anaphylactic reaction. However, the injection of 10PEG−DODAP−liposome showed one lethal anaphylaxis case and one mild anaphylaxis case. Antisera obtained from the animal sensitized as described above were inoculated (0.05 mL) intradermally into fresh guinea pigs. The presence of passive cutaneous anaphylaxis was evaluated after intravenous injections (1 mL/kg) of three liposomes with Evans blue. No dye leakage was detected at any inoculated skin point for PEG−liposome or liposome, but a slight leakage was detected in one inoculated skin point for 10PEG−DODAP−liposome. These results indicate the absence of acute allergic reactions at repeated injections of HbVs despite the anti-PEG antibody induction. Not all the PEG-modified liposomes show anaphylaxis, and it may depend on the amount of PEGylated phospholipid and lipid composition of PEG-modified liposomes.



1. INTRODUCTION

Hemoglobin-based oxygen carriers (HBOCs) of various types have been developed for decades to mitigate or avoid difficulties posed by the present blood donation and transfusion system.[1,2] Such difficulties include the possibilities of transfusion-related transmission of disease, blood-type mismatching, fluctuating numbers of blood donors being affected by social events such as an aging population and COVID-19, and limitations for logistics and stockpiling imposed by the short shelf life of blood products. One HBOC, the Hb vesicle (HbV), encapsulates a concentrated and purified human Hb in a phospholipid vesicle (liposome). The safety and efficacy of the fluid of HbV [(Hb) = 10 g/dL] as a transfusion alternative and as an agent for oxygen therapeutics and other indications have been studied extensively.[3−8] The HbV surface is covered with polyethylene glycol (PEG) by incorporating only 0.3 mol % of PEG-conjugated phospholipid, which is important for a long-term storage of HbV in a deoxygenated state[9] and for homogeneous distribution in plasma phase without inducing aggregation after intravenous administration.[10]

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The liposomal membrane of HbV is highly biocompatible. In fact, it does not induce complement activation in rats[11] nor activate complement in fresh human serum in vitro. In addition, no anaphylactic reaction was observed after repeated injections into rats.[12] However, anti-PEG IgM is produced by HbV injection into mice, resulting in facilitated HbV clearance at a low dosage but not when used at clinically relevant larger dosages as a blood substitute.[13] Other than the shortened circulation half-life, that study did not specifically examine anaphylactic reactions.

Even though PEGylated surface is biocompatible and PEGylation applied widely for nanomedicines to prolong its circulation persistence during clinical use, recent reports have described the presence of pre-existing anti-PEG antibodies in human,[14,15] probably because of exposure to PEGylated materials (such as detergents and cosmetics) in daily life, in addition to injection of PEGylated drug carriers.[16,17] The presence of anti-PEG antibodies leads to a shorter circulation time of administered PEGylated materials and apprehension of various undesirable allergic reactions.[18]

A phase study of pegnivacogin, an RNA aptamer conjugated with a 40 kDa branched molecule of methoxy PEG, was conducted as an inhibitor for coagulation factor IXa. Reportedly, this PEG-conjugated RNA induced the first-exposure allergic reactions in 3 of 640 patients, probably because of the pre-existing anti-PEG antibodies.[14] In that paper, the authors suggest testing for pre-existing anti-PEG antibodies during clinical trials of PEGylated materials, especially those containing PEG at high densities, or which are given at doses that would expose patients to large amounts of PEG. In addition, it is now widely discussed that the rare incidence of the anaphylactoid reactions in response to the mRNA COVID-19 vaccines might be attributable to the components of PEG derivatives in those vaccines,[19−21] although the mechanism remains controversial.

The first-in-human phase 1 clinical trial of HbV injecting healthy adult male volunteers showed the presence of pre-existing anti-PEG IgG in the volunteers of cohort #3 (n = 3), though the incidence of infusion reaction was not necessarily related to the presence of the pre-existing antibody.[22] However, considering that HbV is a PEGylated investigational product, adverse reactions, especially acute anaphylactic reaction that might be caused by anti-PEG antibodies in the bloodstream, need to be seriously concerned.

For this study, we used two sets of experiments to reconfirm the absence of anaphylactic reactions. The first experiment uses rats with multiple injections of HbV generating anti-PEG IgM. This preclinical study was conducted according to good laboratory practice (GLP) regulations. The second experiment includes examination of Hartley guinea pigs, which are used widely for immunogenicity studies[23,24] to evaluate the absence of active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA), to scrutinize the immunogenicity profile of PEGylated liposomes used for HbV.

2. MATERIALS AND METHODS

2.1. Preparation of HbV and Related PEGylated (PEG−Liposome) and Unmodified Liposomes (Liposome). Under sterile conditions at the Department of Chemistry, Nara Medical University, HbVs were prepared as described in an earlier report.[4] From outdated donated blood provided by the Japanese Red Cross (Tokyo, Japan), human carbonylhemoglobin (HbCO) solution was purified via carbonylation, pasteurization, nanofiltration, dialysis, and ultrafiltration to 40 g/dL. Subsequently, pyridoxal 5′-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 1. The lipid bilayer of the vesicles comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG; Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (DPSE−PEG; NOF Corp., Tokyo, Japan) at a molar composition of 5/4/0.9/0.03. The content of DSPE−PEG is only 0.3 mol %. After encapsulation of the HbCO solution into the vesicle using a kneading method,[25] HbCO was converted to oxyhemoglobin using a photodissociation procedure by illuminating visible light in an aerobic condition. The encapsulation efficiency reached nearly 60%. The unencapsulated Hb is removed out by ultrafiltration, and the remaining unencapsulated Hb was less than 2%. Finally, the suspension was deoxygenated to prevent autoxidation during long-term storage. The Hb concentration in the suspension was adjusted to 10 g/dL. The particle size distribution, 250 ± 50 nm, was measured using a light-scattering method (nanoparticle analyzer, model SZ-100; HORIBA Ltd., Kyoto, Japan). The sterility of the suspension was confirmed by Charles River Endotoxin and Microbial Detection Singapore Pte. Ltd.

For antigenicity testing of the liposomes using guinea pigs, xenogeneic human Hb was not encapsulated. The PEG-modified liposome suspension, defined as "PEG−liposome", was prepared under sterile conditions using the same lipid composition as that shown above. The PEG-unmodified liposomes, defined as "liposome", were prepared without mixing DSPE−PEG. For comparison, 10 times excessively PEG-modified liposome with an ionizable lipid, 1,2-dioleoyloxy-3-(dimethylamino)propane (DODAP) was prepared with the lipid composition of DPPC/cholesterol/DODAP/DSPE−PEG = 5/4/2/0.3, defined as "10PEG−DODAP−liposome". DODAP was purchased from Toronto Research Chemicals (Toronto, Canada). DODAP is a tertiary amine known to become cationic at a lower pH and is utilized for mRNA transfection techniques. The lipid powders were dispersed in saline and were extruded through the membrane filters (nitrocellulose membrane, pore sizes 3.0, 1.0, 0.6, 0.45, 0.33, and 0.22 μm; Merck Millipore Ltd., Cork, Ireland) in a step-by-step manner for particle size regulation. The average particle size was 313−374 nm. The lipid concentration was adjusted to about 6 g/dL. For the PCA reaction study, Evans blue (lot #SHBL1996; Sigma-Aldrich Corp.) was dissolved in a liposome suspension at a concentration of 10 mg/mL.

2.2. Animal Experiment Ethics and Regulations. The Laboratory Animal Care and Use Committee of Nihon Bioresearch Inc. approved all experimental protocols. The protocols comply with the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare, Japan (notification no. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, Japan, June 1, 2006) in accordance with the Declaration of Helsinki and the Regulations of the Committee for the Ethical Treatment of Animals (Nihon Bioresearch, April 2, 2007). The rat study complied with the GLP regulation, the Ministerial Order on Standards for Non-Clinical Studies Related to Safety of

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Pharmaceuticals (Order of the Ministry of Health and Welfare no. 21 of 1997).

2.3. Procedures for Rat Study. Experiments were conducted using 17 male SD rats (6 weeks old, 206−215 g bw). They were purchased from Charles River Laboratories Japan (Hino, Japan) and were used after a quarantine period of 5 days and habituation for 3 days (Figure 1). The rats were

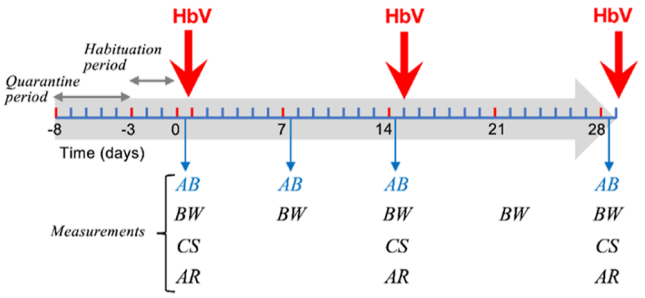

Figure 1. Experiment schedule of repeated HbV injections and measurements. AB, antibody; BW, bodyweight; CS, clinical sign; AR, anaphylactic reaction. On each day of injection, clinical sign was confirmed before and soon after the injections. On other days, observation was performed once a day.

grouped randomly into three groups to create a homogeneous distribution of the average bodyweight by using a computer program (IBUKI; Nihon Bioresearch Inc.). Other animals were sacrificed using an isoflurane overdose and phlebotomy. The rats were housed in cages (W.240 × D.380 × H.200 mm) and were provided with food and water ad libitum in a ventilated, temperature-controlled and humidity-controlled room on a 12 h dark/light cycle.

A rat was inserted into a Bollman cage (Natsume Seisakusho Co., Ltd., Tokyo, Japan). Then, the test fluid was injected from the tail vein through a 24G indwelling needle (Terumo Corp., Tokyo, Japan) and extension tubing connected to a 20 mL polypropylene syringe (Terumo Corp.) filled with a prefiltered test fluid. The syringe was set onto a pumping machine (SP-110, SP-115; JMS Co. Ltd.). The test fluid was injected at a rate of 1.5 mL/kg/min. The first group received HbV at a dose rate of 16 mL/kg (n = 5). The second group received 32 mL/kg (n = 5). The control group (n = 5) received 32 mL/kg saline solution (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan). The rats received the same fluid at the same dosage on days 15 and 29. Thereby, the rats received injections three times at 2 week intervals. On each day of injection, the performance status and the presence of any abnormal physiological sign were confirmed before and soon after the injections. On other days, observation was performed once a day. The bodyweight was measured on days 1, 8, 15, and 29 using an electric balance (PB3002-S/FACT; Mettler Toledo International Inc.).

2.4. Measurement of Plasma Anti-PEG Antibodies in Rats. On days 1, 15, and 29 immediately before injection of the test fluid and on day 8, about 0.3 mL of blood was withdrawn from the jugular vein without anesthesia using a heparinized 23G needle connected with a 1 mL syringe. Blood was gently mixed with a solution of a high molecular weight dextran solution (dissolved in saline at 20 w/v %, M_r 450−650 kDa; Sigma) at a ratio of 9:1 by volume. Then, it was centrifuged at 3000 rpm (2150g) for 15 min to remove blood cells and HbV as precipitates.[26] The supernatant plasma fraction was used for the antibody measurements using the Rat Anti-PEG IgG ELISA kit (PEGG-2) and Rat Anti-PEG IgM ELISA kit (PEGM-2) (Life Diagnostics, Inc., West Chester, USA), containing a PEG−BSA-coated 96-well plate and antirat IgM or IgG HRP conjugates, reference standards, and a 3,3′,5,5′-tetramethylbenzidine ready-to-use solution. All procedures were described in the instruction manuals (https://lifediagnostics.com). The unit for the concentration, units per milliliter, is defined by the manufacturer.

2.5. Observation of Anaphylactic Reactions in Rats. The presence or absence of anaphylactic reactions was observed in terms of fluffiness, tear, and respiration abnormalities on days 1, 15, and 29, at 1 h after every injection of HbV or saline with 2 weeks of intervals. Reportedly, immunized rats with subcutaneous ovalbumin injection in combination with Freund's complete adjuvant (FCA) show lethal anaphylactic reactions by intravenous ovalbumin injection after a 2 week interval.[12,27,28]

2.6. Statistical Analysis of Rat Experiments. All data were expressed as the mean ± standard deviation. The statistical significance of the difference between the groups was determined using Bartlett's test of homogeneity of variance, followed by Dunnett's multiple comparison test. When homogeneity of variance was not confirmed, Steel's test was conducted. $p < 0.05$ and 0.01 were inferred as statistically

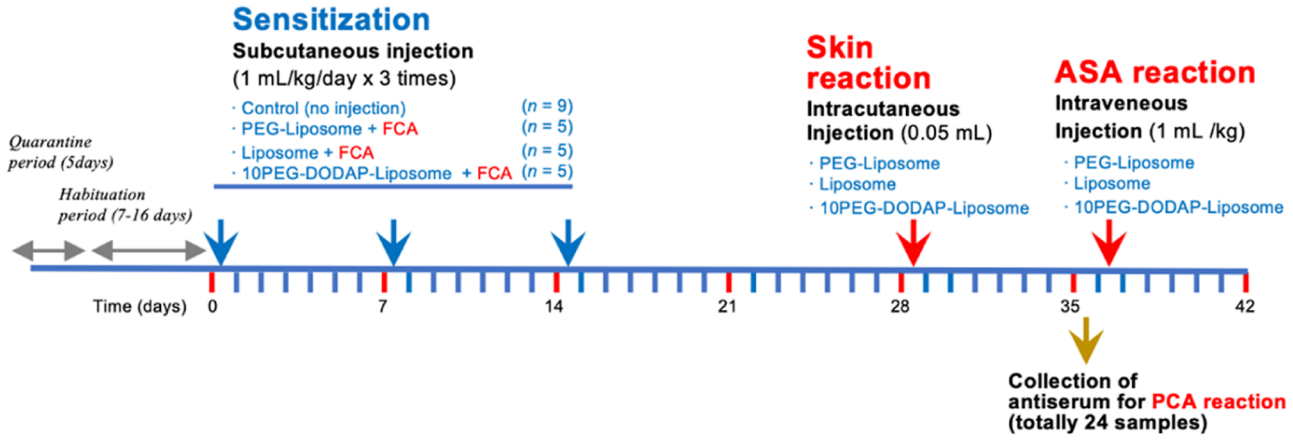

Figure 2. Schedule of guinea pig experiment of sensitization by liposome injections, observation of ASA, and antisera collection for the observation of PCA.

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significant. Statistical analysis was not conducted for the observation of anaphylactic reactions.

2.7. Procedures for Guinea Pig Study. Experiments were conducted using male guinea pigs (Kwl: Hartley) purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan), which were used after a quarantine period of 5 days and subsequent habituation for 7 or 16 days. During that period, bodyweight measurements were taken at least three times. General observations were conducted every day. Animals with no abnormalities were used for experiments. On the day of sensitization, 16 animals (5 weeks old) were grouped randomly using the IBUKI computer program to produce a homogeneous distribution of the average bodyweight. For PCA observation, 10 animals (7 weeks old) were prepared. The animals were housed in cages (W.350 × D.580 × H.350 mm) with a maximal capacity of five animals. They were housed individually after antigen challenging. The animals were provided with food and water ad libitum in a ventilated temperature-controlled and humidity-controlled room on a 12 h dark/light cycle.

FCA (lot 0189600; Difco Laboratories) was obtained from Nippon Becton Dickinson Co., Ltd. (Tokyo, Japan). The liposome suspensions were mixed with an equal volume of FCA for sensitization. The test material was put in a disposable polypropylene syringe with a 23G needle (Terumo Corp., Tokyo, Japan) and was injected three times subcutaneously (1 mL/kg/days × 3) at 1 week intervals. The first group received PEG−liposome + FCA (n = 5). The second group received liposome + PCA (n = 5). The third group received 10PEG−DODAP−liposome plus FCA (n = 5). The control group received no injection (n = 9) (Figure 2). Two weeks later, intracutaneous injection of liposome, PEG−liposome, or 10PEG−DPDAP−liposome was carried out for skin reaction test. Three weeks later (1 day before the ASA reaction observation), a blood specimen (2.5 mL) from each animal (24 animals in all) was collected by cardiopuncture without anesthesia into a vacuum blood collection tube for the preparation of antiserum for the PCA experiment. After the blood was centrifuged (4 °C, 3000 rpm, 15 min), the antiserum was stored in a freezer (−90 to −70 °C).

2.8. Skin Reaction of Guinea Pigs. Observation of the skin reaction was performed 14 days after the final sensitization injection. The back skin of the animal, which had been shaved 1 day prior, was injected in one or two spots per animal with PEG−liposome, liposome, or 10PEG−DODAP−liposome (0.05 mL) using a Myjector syringe (Terumo Corp.). The skin reaction was evaluated before and soon after the injection and at the time points of 24 ± 2, 48 ± 2, and 72 ± 2 h after the injection. The reactions of erythema and eschar formation and edema formation were scored from 0 (none) to 4 (strong). The sum of the scores for each animal was averaged.

2.9. ASA Reaction of Guinea Pigs. Observation of the ASA reaction was performed 22 days after the final sensitization. PEG−liposome, liposome, or 10PEG−DODAP−liposome was put in a disposable polypropylene syringe (Terumo Corp.) with a needle (Natsume Seisakusho Co., Ltd.) and was injected into an auricular vein (1 mL/kg). The body temperature was measured before injection and 1 h after injection. The presence or absence of ASA reactions was monitored within 30 min, as was death within 24 h. The observation items included nose-licking or rubbing, fur erection, weak or diminished muscle tone, labored breathing, sneezing and/or coughing, retching, rales, convulsion, prostration, and death. When the number of positive items was 1−3, the reaction was judged as "mild". When the number of positive items was 4−7, it was judged as "moderate". When the number was 8 and higher or the animal died, it was judged as "severe". After observation, the animals were euthanized by bleeding from the abdominal aorta under isoflurane anesthesia.

2.10. PCA Reaction of Guinea Pigs. The stored antisera obtained from 16 animals were used for inoculation. The back skins of fresh guinea pigs were shaved; several antisera were inoculated per animal (0.05 mL per spot) using Myjectors. Two fresh animals were used for the inoculation of each antiserum.

Four hours after inoculation, PEG−liposome, liposome, or 10PEG−DODAP−liposome dissolved with Evans blue was put in a disposable polypropylene syringe (Terumo Corp.) with a needle (Natsume Seisakusho Co., Ltd.) and was injected into an auricular vein (1 mL/kg).

Thirty minutes after the intravenous injection, the animals were euthanized by bleeding from the abdominal aorta under isoflurane anesthesia. The back skin was peeled carefully. From the back side of the skin, blue macula caused by the vascular leakage of Evans blue was observed. It was inferred as tested positive when the average macula diameter of the two animals exceeded 5 mm for each spot of antiserum inoculation. When the PCA reaction was tested positive, the antiserum was diluted (×100, ×300, ×1000, and ×3000). Then, inoculation and intravenous injection of liposomal injections were repeated using fresh animals until the PCA reaction was tested as negative. PCA titer was defined as the dilution factor to be tested negative. When the PCA reaction was tested negative without dilution, the PCA titer was judged as <1.

2.11. Measurement of Serum Anti-PEG Antibodies in Guinea Pigs. The stored antisera obtained from 16 animals were used for the measurement. Goat antiguinea pig IgM HRP conjugate (Goat, polyclonal) was purchased from LifeSpan BioSciences, Inc. (MA, USA). Goat antiguinea pig IgG HRP conjugate was purchased from Bethyl Laboratories, Inc. (TX, USA). Briefly, a 96-well plate was coated with 10 nmol of $mPEG_{2000}$−DSPE for 2 h and then blocked with 1% BSA/Tris-buffered saline (blocking buffer) for 1 h. After washing three times with 0.05% CHAPS/Tris-buffered saline (washing buffer), serum samples diluted in blocking buffer were added to each well. After 1 h incubation, the plate was washed five times. Goat antiguinea pig IgM HRP conjugate or Goat antiguinea pig IgG HRP conjugate was added and incubated for 1 h. After washing five times, 3,3′,5,5′-tetramethylbenzidine was added, and the reaction was stopped using 2 M H_2SO_4. The absorbance of each well was measured at 450 nm using a microplate reader.

3. RESULTS

3.1. Observations of Physiological Signs and Bodyweight in Rat Experiments. All the rats receiving multiple HbV or saline injections survived until intentional euthanization at 29 days. Especially, the high-dose HbV group tolerated repeated injections of 96 mL/kg volume, which corresponds to 1.7 times greater than the whole blood volume (56 mL/kg).

After every injection of HbV, the rats in both groups showed skin reddening at auricles and limbs of less hairs because of the color and small particle size of HbV present in subcutaneous capillaries. Some rats showed pale reddish urine because a small amount of unencapsulated Hb (<2%) was present in the HbV suspension injected.

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The bodyweight of the low-dose HbV (16 mL/kg) group increased from 283 ± 4 to 449 g at day 29 without significant differences to the control saline group (Figure 3). However, the high-dose HbV (32 mL/kg) group was found to have significantly lower values on days 8 and 22.

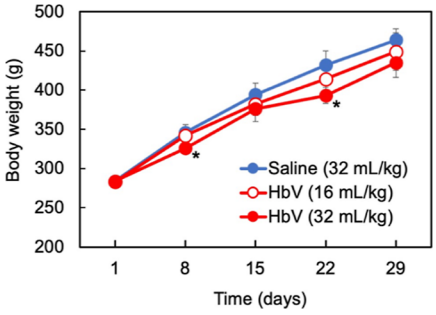

Figure 3. Changes in the bodyweight of SD rats during repeated injections. Mean ± SD. *Significantly different from the control group ($P < 0.01$ by Dunnett's test).

3.2. Measurement of Plasma Anti-PEG Antibodies in Rats. The level of plasma anti-PEG IgM before the first injection was below the detection limit for all groups. For the high-dose HbV group (32 mL/kg), all rats showed the presence of anti-PEG IgM on day 8 with 191.6 ± 88.7 U/mL (Table 1 and Figure 4). On day 15 before injection, three rats among five showed anti-PEG IgM with 278.0 U/mL, on the average of five rats. On day 29 before injection, three rats showed anti-PEG IgM with 51.9 ± 47.5 U/mL. For the low-dose Hb-V group (16 mL/kg) on day 8, only three rats showed anti-PEG IgM with 82.4 ± 90.6 U/mL. On day 15 before injection, three rats showed anti-PEG IgM with 156.3 ± 179.0 U/mL. On day 29 before injection, two rats showed anti-PEG IgM with 43.7 ± 63.1 U/mL. The level of anti-PEG IgM reached its maximum on day 15. It then tended to decrease on day 29. Because of the large deviation, statistical significance in comparison to the control group was confirmed only on day 8. The level of anti-PEG IgM for the control saline group was below the detection limit at all time points. The level of plasma anti-PEG IgG was below the detection limit for all groups at all time points.

3.3. Observation of Anaphylactic Reactions in Rats. The results obtained for observations of the anaphylactic reaction are presented in Table 2. No rat of any group showed any anaphylactic reaction, fluffiness, tear, and respiration abnormalities at any time point of repeated HbV injections.

3.4. Observation on Skin Reactions of Guinea Pigs. The control animals with no sensitization injection showed no skin reaction after intracutaneous injection (0.05 mL) of either PEG−liposome or liposome at any observation time point (Table 3). The animals sensitized with either PEG−liposome + FCA or liposome + FCA showed no skin reaction immediately after intracutaneous injection (0.05 mL) of PEG−liposome or liposome, respectively. However, at 24, 48, and 72 h after the injections, the animals developed erythema and eschar formation and edema formation with mean skin reaction (scores 4.4−5.2). No apparent difference was observed between sensitization agents of the PEG−liposome and liposome.

On the other hand, 10PEG−DODAP−liposome showed different profiles. The control animals with no sensitization showed slight erythema and eschar formation (score 1) at 24, 48, and 72 h after injections of 10PEG−DODAP−liposome.

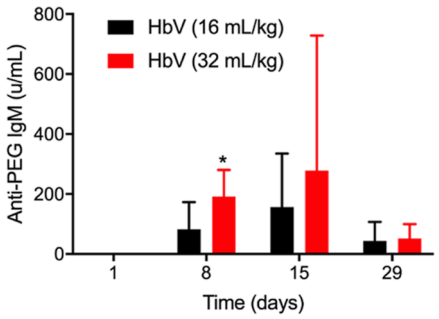

Figure 4. Anti-PEG IgM concentrations (U/mL) in rat plasma after intravenous administration of HbV. Significantly different from the control group (<0.05 by Steel's test).

Table 2. Observations of Anaphylactic Reactions after Every Injection of HbV (16, 32 mL/kg) or Saline (32 mL/kg)

		number of animals with abnormality		
group	reactions	1 day	15 days	29 days
control saline (32 mL/kg) (n = 5)	fluffiness	0	0	0
	tear	0	0	0
	respiratory abnormality	0	0	0
HbV (16 mL/kg) (n = 5)	fluffiness	0	0	0
	tear	0	0	0
	respiratory abnormality	0	0	0
HbV (32 mL/kg) (n = 5)	fluffiness	0	0	0
	tear	0	0	0
	respiratory abnormality	0	0	0

Table 1. Anti-PEG IgM Concentrations (U/mL) in Rat Plasma

	anti-PEG IgM (U/mL) after 16 mL/kg HbV injections					anti-PEG IgM (U/mL) after 32 mL/kg HbV injections			
animal number	1 day	8 days	15 days	29 days	animal number	1 day	8 days	15 days	29 days
#16-1	0.0	219.3	349.8	137.5	#32-1	0.0	322.7	197.0	0.0
#16-2	0.0	104.8	347.6	0.0	#32-2	0.0	164.1	0.0	0.0
#16-3	0.0	0.0	0.0	0.0	#32-3	0.0	237.9	1069.0	80.5
#16-4	0.0	0.0	0.0	0.0	#32-4	0.0	108.6	0.0	88.1
#16-5	0.0	88.1	84.0	81.2	#32-5	0.0	124.7	123.9	90.9
mean(n = 5)	0.0	82.4	156.3	43.7	mean (n = 5)	0.0	[a]191.6	278.0	51.9
SD	0.0	90.6	179.0	63.1	SD	0.0	88.7	450.2	47.5

[a]Significantly different from the control group (<0.05 by Steel's test). Values less than the quantification limit were regarded as 0.0 U/mL.

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Table 3. Observations of Guinea Pig Skin Reactions

			skin reaction average score				
					time after injection (h)		
animal number	sensitization	test substance (0.05 mL, s.c.)	before injection	immediately after injection	24	48	72
M01101−M01106	none treated (n = 6)	PEG−liposome	0	0	0	0	0
		liposome	0	0	0	0	0
M02201−M02205	PEG−liposome + FCA 1 mL/kg/day × three times s.c. (n = 5)	PEG−liposome	0	0	5.2 ± 1.1	4.8 ± 1.1	4.8 ± 1.1
		liposome	0	0	5.2 ± 1.1	4.8 ± 1.1	4.8 ± 1.1
M03301−M03305	liposome + FCA 1 mL/kg/day × three times s.c. (n = 5)	PEG−liposome	0	0	4.4 ± 0.9	4.4 ± 0.9	4.4 ± 0.9
		liposome	0	0	4.4 ± 0.9	4.4 ± 0.9	4.4 ± 0.9
M01401−M01403	none treated (n = 3)	10PEG−DODAP	0	0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
M02501−M02505	10PEG−DODAP−liposome + FCA 1 mL/kg/day × three times s.c. (n = 5)	10PEG−DODAP	0	0	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0

Table 4. Observations of ASA of Sensitized Guinea Pigs after Intravenous Injection of PEG−Liposome, Liposome, or 10PEG−DODAP-Liposome Suspension

			symptom[a]											body temperature (°C)	
animal number	sensitization	challenged antigen i.v	1	2	3	4	5	6	7	8	9	10	judgment[b]	before challenge	1 h after challenge
M01101	none treated	PEG−liposome (1 mL/kg)	−	−	−	−	−	−	−	−	−	−	0	38.5	38.4
M01102			−	−	−	−	−	−	−	−	−	−	0	38.8	38.2
M01103			−	−	−	−	−	−	−	−	−	−	0	38.5	38.0
M02201	PEG−liposome + FCA 1 mL/kg/day × three times s.c.		−	−	−	−	−	−	−	−	−	−	0	38.8	39.1
M02202			−	−	−	−	−	−	−	−	−	−	0	38.1	38.0
M02203			−	−	−	−	−	−	−	−	−	−	0	37.9	38.3
M02204			−	−	−	−	−	−	−	−	−	−	0	38.6	39.2
M02205			−	−	−	−	−	−	−	−	−	−	0	38.5	38.7
M01104	none treated	liposome (1 mL/kg)	−	−	−	−	−	−	−	−	−	−	0	37.8	37.7
M01105			−	−	−	−	−	−	−	−	−	−	0	38.7	38.3
M01106			−	−	−	−	−	−	−	−	−	−	0	38.5	38.8
M03301	liposome + FCA 1 mL/kg/day × three times s.c.		−	−	−	−	−	−	−	−	−	−	0	38.5	38.7
M03302			−	−	−	−	−	−	−	−	−	−	0	38.3	38.5
M03303			−	−	−	−	−	−	−	−	−	−	0	38.6	38.6
M03304			−	−	−	−	−	−	−	−	−	−	0	38.4	38.8
M03305			−	−	−	−	−	−	−	−	−	−	0	38.6	38.5
M01401	none treated	10PEG−DODAP−liposome (1 mL/kg)	−	−	−	−	−	−	−	−	−	−	0	38.7	39.1
M01402			−	−	−	−	−	−	−	−	−	−	0	38.7	38.9
M01403			−	−	−	−	−	−	−	−	−	−	0	38.8	38.3
M02501	10PEG−DODAP−liposome + FCA 1 mL/kg/day × three times s.c.		−	−	−	−	−	−	−	−	−	−	0	39.0	39.3
M02502			−	−	−	−	−	−	−	−	−	−	0	38.6	38.9
M02503			−	−	−	−	−	−	−	−	−	−	0	38.7	39.3
M02504			+	+	+	−	+	−	−	−	−	−	II	38.8	38.0
M02505			+	+	−	+	+	−	−	+	−	+	III	38.5	

[a](1) nose-licking or rubbing, (2) fur erection, (3) weak or diminished muscle tone, (4) labored breathing, (5) sneezing or coughing, (6) retching, (7) rales, (8) convulsion, (9) prostration, and (10) death; −, negative; +, positive. [b]0, no symptoms were observed; I, number of symptoms were 1−3; II, number of symptoms were 4−7; III, number of symptoms were over 8; or the animal died.

The animals sensitized with 10PEG−DODAP-liposome showed no skin reaction immediately after the intracutaneous injection of 10PEG−DODAP-liposome, but stronger reactions (score 6) were observed at 24, 48, and 72 h.

3.5. Observation of ASA Reactions of Guinea Pigs.

Neither the PEG−liposome + FCA-sensitized nor liposome + FCA-sensitized animals showed any anaphylactic reaction after intravenous injections of PEG−liposome or liposome

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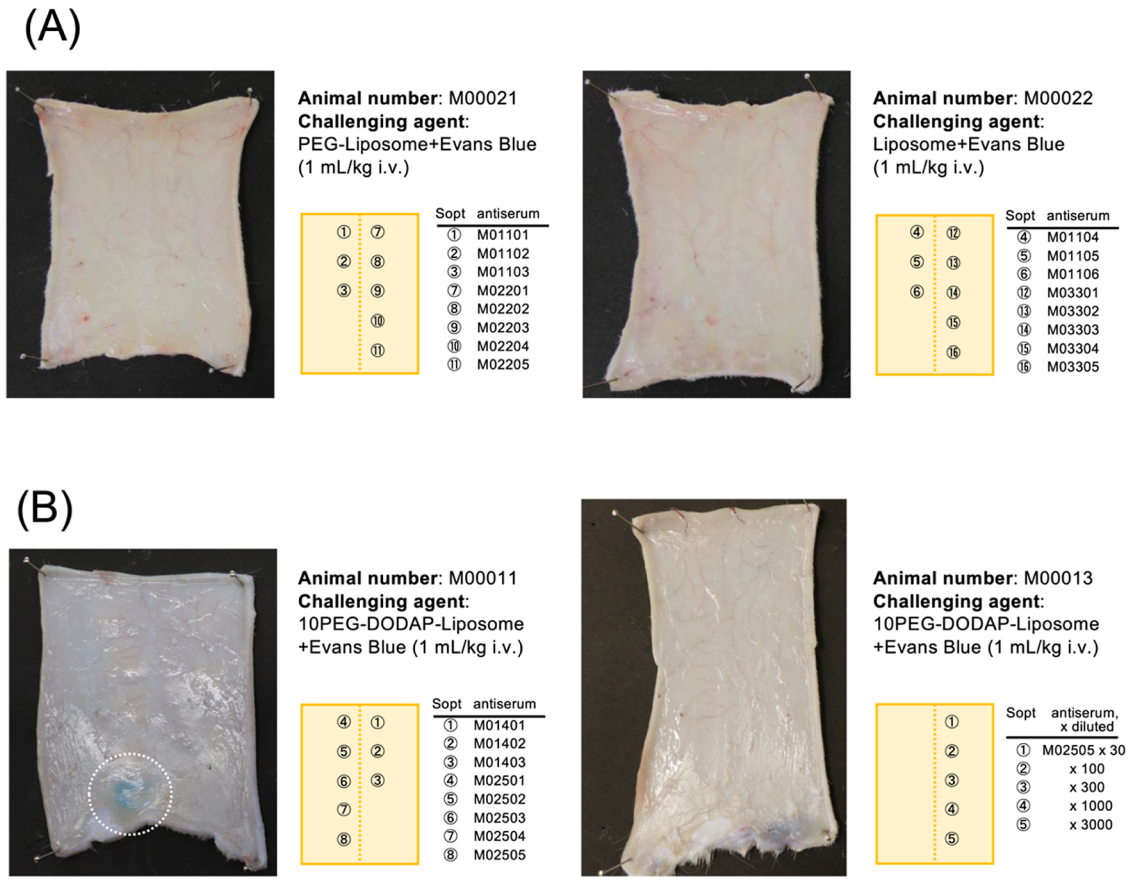

Figure 5. Observations of passive cutaneous reactions (PCAs). (A) Inoculated spots with antisera obtained from PEG−liposome- or liposome-injected animals (M00021−M00024) showed no leakage of Evans blue after intravenous injection of PEG−liposome or liposome with Evans blue to fresh animals (pictures; M00021 and M00022). (B) (Left) Inoculated spots with antisera obtained from 10PEG−DODAP-liposome-injected animals showed one case of Evans blue leakage after intravenous injection of 10PEG−DODAP-liposome with Evans blue into one fresh animal M00011, but no leakage can be seen in another animal M00012 (picture not shown). The test positive antiserum was derived from M02505. (Right) Inoculation of the diluted M02505 antisera (×30, ×100, ×1000, and ×3000) to two fresh animals (M00013 and M00014) showed no leakage of Evans blue after i.v. injection of 10PEG−DODAP-liposome (Picture, M00013). Therefore, the PCA titer was judged 1. Animal numbers (M0xxxx) correspond to those in Tables 3−5.

suspension, respectively (Table 4). The score was judged as 0. The body temperature did not tend to increase.

On the other hand, two of the five 10PEG−DODAP−liposome + FCA-sensitized animals showed an anaphylactic reaction. One animal (no. M02504) showed judgment II, and the other animal (no. M02505) died 4 min after injection and showed judgment III.

3.6. Observation of PCA Reactions of Guinea Pigs. In the case of PEG−liposome and liposome, all inoculated spots of 16 antisera on the back skins of four animals showed no leakage of the Evans blue dye (Figure 5A and Table 5) after intravenous injection of PEG−liposome or liposome suspensions mixed with Evans blue. Because no dilution of antisera was necessary for any antiserum, the PCA titer was scored <1 for all antisera.

On the other hand, in the case of 10PEG−DODAP−liposome-sensitized animals, one spot inoculated with antiserum obtained from the animal (no. M02505) with strong anaphylaxis (death) showed slight leakage of the Evans blue dye after intravenous injection of 10PEG−DODAP−liposome mixed with Evans blue (Figure 5B). Accordingly, the antiserum from no. M02505 was diluted ×30, ×100, ×300, ×1000, and ×3000, and the experiment was repeated. No leakage was observed for any dilution. Therefore, the PCA titer was scored 1.

3.7. Measurement of Serum Anti-PEG Antibodies in Guinea Pig. Serum derived from animals sensitized with PEG−liposome or 10PEG−DODAP−liposome was positive for not only anti-PEG IgM but also anti-PEG IgG (Figure 6). In contrast, serum derived from animals sensitized with liposome failed to do so. Unexpectedly, while the sera derived from nontreated animals (M01101−M01106) which received intracutaneous injection of PEG−liposome for skin reaction test 1 week before sample collection were negative for anti-PEG antibody, the sera derived from nontreated animals (M01401−M01403) which received intracutaneous injection of 10PEG−DODAP−liposome for skin reaction test were found positive. Therefore, only the former sera were considered as control sera for antibody measurement. What caused the difference between the former and the latter will be discussed elsewhere in the Discussion Section.

4. DISCUSSION

This rat study was conducted as a GLP preclinical study of HbV from the toxicological perspective in terms of its immunogenicity before starting a phase 1 clinical trial.[5,22] Our study confirmed the absence of anaphylactic reactions at

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Table 5. Observation of PCA of Sensitized Guinea Pigs after Intravenous Injection of PEG−Liposome, Liposome, or 10PEG−DODAP-Liposome Suspension with Evans Blue

	inoculated antiserum (0.05 mL per spot)			
fresh animal number	sensitization	source (animal number)	challenged antigen i.v	PCA titer[a]
M00021, M00023	none treated	M01101	PEG−liposome + Evans blue (1 mL/kg)	<1
		M01102		<1
		M01103		<1
	PEG−liposome + FCA 1 mL/kg/day × three times s.c.	M02201		<1
		M02202		<1
		M02203		<1
		M02204		<1
		M02205		<1
M00022, M00024	none treated	M01104	liposome + Evans blue (1 mL/kg)	<1
		M01105		<1
		M01106		<1
	liposome + FCA 1 mL/kg/day × three times s.c.	M03301		<1
		M03302		<1
		M03303		<1
		M03304		<1
		M03305		<1
M00011, M00012[b]	none treated	M01401	10PEG−DODAP−liposome + Evans blue (1 mL/kg)	<1
		M01402		<1
		M01403		<1
	10PEG−DODAP−liposome + FCA 1 mL/kg/day × three times s.c.	M02501		<1
		M02502		<1
		M02503		<1
		M02504		<1
		M02505		1

[a]PCA titer is defined as the dilution factor to be tested negative. When the PCA reaction is tested negative without dilution, the PCA titer is judged as <1. [b]For diluted antisera inoculation, two fresh animals, M00013 and M00014, were additionally utilized.

the repeated HbV injections. Moreover, a guinea pig study was conducted, from which we reconfirmed the absence of anaphylactic reactions from conventional immunogenicity testing of ASA and PCA reactions.

In the rat study, we confirmed the absence of anaphylactic reactions at the repeated PEG−liposome (HbV) injections with 2 week intervals, even though anti-PEG IgM, but not anti-PEG IgG, was detected. This is consistent with our previous study, where HbV showed a slightly accelerated blood clearance phenomenon at a clinically relevant large dosage and anti-PEG IgM induction in rats;[29] however, other than the ABC phenomenon, no anaphylactic reaction was observed. This suggested that PEG derivatives on HbV may not cause an allergic reaction even in the presence of anti-PEG IgM antibody.

We further addressed allergenic potential of the liposome, PEG−liposome, and 10PEG−DODAP−liposome using guinea pigs which were presensitized three times with liposome, PEG−liposome, or 10PEG−DODAP−liposome along with FCA, respectively. Skin reaction tests showed that none of these liposomes induced early-phase skin reaction (Table 3). Unexpectedly, late-phase skin reactions were observed in all types of liposomes with strongest reaction by 10PEG−DODAP liposome. It seems that priming was necessary to induce the skin reaction, and at least lipid components worked as the antigen responsible for the reactions because even the liposome which contained no PEG-conjugated phospholipid induced late-phase skin reactions, and no skin reaction was observed in nontreated animals (Table 3, M01101−M01106). Based on these results, it might be possible that antibodies against lipid component were generated and were involved in the late-phase skin reaction. In fact, the presence of antibodies not only to cholesterol but also to phospholipids, which are related to atherosclerosis or aging, was reported.[30] However, the late-phase reaction is not in the scope of this study.

Referring to the antibody response to PEG derivatives, as was expected, guinea pigs sensitized with PEG−liposome or 10PEG−DODAP−liposome along with FCA became positive for not only anti-PEG IgM but also anti-PEG IgG (Figure 6). However, unexpectedly, elevations of anti-PEG IgG or anti-PEG IgM were observed in nontreated animals (no. M01401−M01403). The following explanation might be put forward. They underwent 10PEG−DODAP−liposome injection (only 0.05 mL) into skin for skin reaction test 2 weeks before ASA reaction test (Figure 2). DODAP is an ionizable lipid and, reportedly, the ionizable lipid could work as an adjuvant through activation of innate immunity and enhancement of cytokine production such as interleukin-1.[31,32] Therefore, while one subcutaneous injection of PEG−liposome did not produce anti-PEG antibodies, one subcutaneous injection of 10PEG−DODAP−liposome might be enough to induce such antibody production. A slightly positive skin reaction (score 1) observed in a nontreated animal (Table 3, M01401−M01403)

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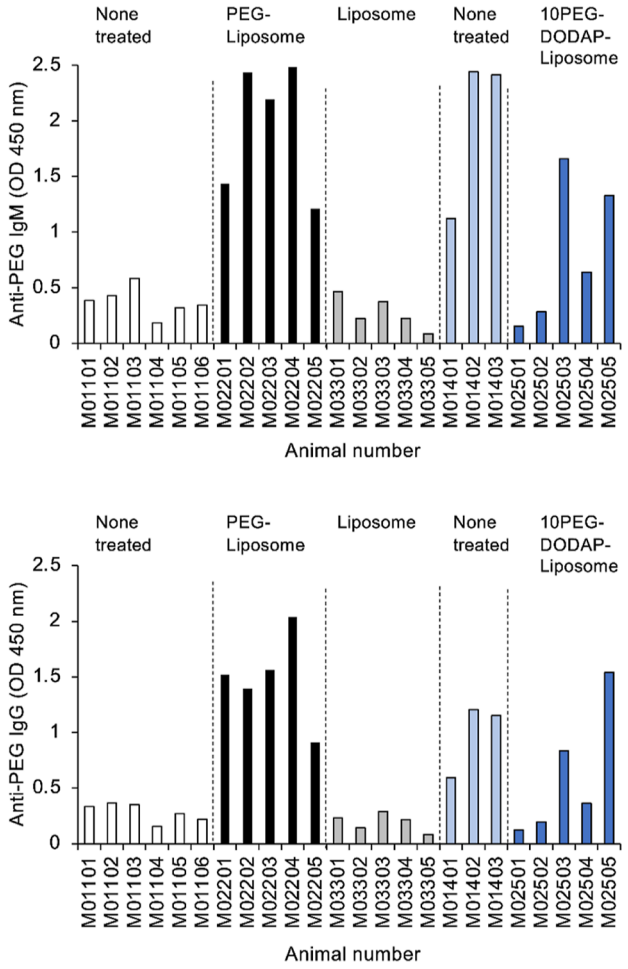

Figure 6. Anti-PEG antibody in guinea pig sera after three times sensitization with liposome, PEG−liposome, or 10PEG−DODAP−liposome along with FCA. The sera derived from animals sensitized with PEG−liposome or 10PEG−DODAP−liposome were positive for both anti-PEG IgM and IgG. The sera derived from nontreated animals (M01101−M01106) were considered as controls negative; however, the sera derived from nontreated animals (M01401−M01403) were positive. The former and the latter received intracutaneous injection of PEG−liposome and 10PEG−DODAP−liposome, respectively, 1 week before the collection of blood sample. Different from PEG−liposome, 10PEG−DODAP−liposome is supposed to be worked as an adjuvant. This may be the reason anti-PEG antibody was produced. Animal numbers (M0xxxx) correspond to those in Tables 3−5.

might reflect local inflammation generated by the above-described biological effects of DODAP.

It should be stressed that intravenous injection of PEG−liposome into animals which were presensitized with PEG−liposome along with FCA showed no systemic anaphylaxis reaction at all (Table 4). In addition, PCA reactions using sera derived from these animals were all negative. Coupling with the result of experiment using rats (Table 2), these results strongly suggested that the PEG derivatives expressed on HbV hardly induce ASA reaction even in the presence of anti-PEG IgG or anti-PEG IgM in the bloodstream (Table 1 and Figure 4). On the contrary, two of five animals that were presensitized with 10PEG−DODAP−liposome along with FCA and challenged with 10PEG−DODAP−liposome did manifest the ASA reaction. One of them (M02505) died of the ASA reaction. In addition, serum from this animal was positive for the PCA reaction. These results indicated that anti-PEG IgE or anti-PEG IgG was responsible for the ASA reaction. However, considering that nontreated animals were also positive for anti-PEG IgG and anti-PEG IgM, and none of them manifested any adverse effect, anti-PEG IgE generated in this animal rather than anti-PEG IgG might be responsible for ASA reaction. Another possibility was that anti-PEG IgG generated through three times sensitization along with FCA had higher affinity to PEG derivatives than antibody unexpectedly generated by only one sensitization without FCA. The high affinity might give the former antibody the stronger potential for triggering anaphylactic reaction than the latter.[33]

It is recently well recognized that the interaction between anti-PEG-antibody and a PEG chain is very weak,[34] which would be the reason for the extremely low rate of anaphylactic reaction in spite of the induction of anti-PEG antibody in many healthy people who are daily exposed to cosmetics and detergents comprising PEG. Therefore, we suppose the density of PEG chains and a secondary interaction force, namely, electrostatic interaction, should help the binding of anti-PEG-antibody to PEG chain. Reportedly, PEGylated ionizable liposomes with DODAP encapsulating oligonucleotide induce anti-PEG-IgM antibodies in mice and cause anaphylactic shock reactions on a second injection of the same liposomes.[35] Similarly, PEGylated ionizable liposomes with 1,2-dioleoyl-3-dimethylaminopropane (DODMA, a similar structure with DODAP) encapsulating plasmid DNA generate strong anti-PEG IgM and anti-PEG IgG responses in mice and cause significant lethal anaphylactic reactions.[36] The result that the 10PEG−DODAP liposome showed anaphylactic reactions in guinea pigs (Table 4) is in line with these reports. The difference of contents between 10PEG−DODAP−liposome and PEG−liposome may explain the outstanding differences between a lethal severe reaction and no reaction, respectively.

It should be noted that the physicochemical properties of liposomes tested in this study are different. First, the amounts of PEG-conjugated phospholipid are different. While 10PEG−DODAP−liposome contained 3 mol % of DSPE−PEG, PEG−liposome contained only 0.3 mol % of DSPE−PEG. This means that the amount of PEG expressed onto one particle of the former is about 10 times that of the latter. Therefore, the latter should interact with a smaller amount of anti-PEG antibodies compared to the former. This may make the complement activation hardly occur on the surface of PEG−liposome even if the antibodies bind to its epitope. It was of interest that complement activation was reportedly occurred by Doxil and anti-PEG antibodies.[37] Considering that Doxil is one of the PEGylated liposomal drugs which contains approximately 5 mol % of DSPE−PEG, this report might support the above-mentioned speculation.

Second, the surface charge is different. While PEG−liposome belongs to anionic liposome because of the presence of DHSG, 10PEG−DODAP−liposome belongs to ionizable (cationic) liposome because of the presence of DODAP. Anti-PEG antibodies and complements can strongly bind to PEGylated cationic liposomes (including 10PEG−DODAP−liposome) compared with PEGylated anionic liposomes (including PEG−liposome) because plasma proteins are anionic[38] and cationic liposomes or nanoparticles are known to be adsorbed with anionic plasma proteins.[39] Therefore, the amount of PEG and the polarity of the liposomal surface, the combination of PEG, and a functional material encapsulated in or embedded on PEGylated cationic liposomes should

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determine the presence and strength of anaphylactic reactions.[34]

The mechanism of anaphylactic reaction after injection of PEG-conjugated materials in relation to the involvement of anti-PEG IgM, IgG, or IgE is still controversial.[40−44] The high incidence of complement activation by IgM should also be involved in the anaphylactic reaction, although HbV did not show complement activation. It is even more difficult to clarify the mechanism of the absence of anaphylactic reactions despite the induction of these antibodies. We plan to investigate this in our ongoing research experimentally and clinically.

5. CONCLUSIONS

We confirmed the absence of anaphylactic reactions in rat at the repeated HbV (PEG−liposome) injections with 2 week intervals, even though the presence of anti-PEG IgM antibody, but not IgG, was detected. Moreover, the guinea pig study was conducted, from which we reconfirmed the absence of anaphylactic reactions to PEG−liposome even in the presence of both anti-PEG IgG and anti-PEG IgM from the conventional immunogenicity testing of ASA and PCA reactions. However, we expect to devote attention to any reaction, including injection reactions, in ongoing clinical studies of HbV because a subject or a patient might have pre-existing anti-PEG antibodies.

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Author Contributions

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Notes

The authors declare the following competing financial interest(s): Of the authors, H.S. is an inventor of patents related to the production and utilization of Hb vesicles.

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ABBREVIATIONS

PEG, polyethylene glycol; HbV, hemoglobin vesicles; ASA, active systemic anaphylaxis; PCA, passive cutaneous anaphylaxis; HBOCs, hemoglobin-based oxygen carriers; GLP, good laboratory practice; HbCO, carbonylhemoglobin; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate; DSPE−PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}; DODAP, 1,2-dioleoyloxy-3-(dimethylamino)propane; FCA, Freund's complete adjuvant

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