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Research Paper

Accelerated Blood Clearance Was Not Induced for a Gadolinium-Containing PEG-poly(L-lysine)-Based Polymeric Micelle in Mice

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Purpose. Accelerated blood clearance (ABC) is induced by repeated injections of PEGylated liposomes. In this study, the ABC was investigated for a gadolinium-containing PEG-poly(L-lysine)-based polymeric micelle (Gd-micelle) and PEGylated liposome (Gd-liposome) in mice.
Materials and Methods. Effects of the first injection of Gd-micelle on the tissue distribution of the second dose of Gd-micelle were studied. Additionally, effects of the first injection of Gd-micelle, Gd-liposome, empty liposome, polyethyleneglycol ($PEG_{500,000}$), and PEG-lipid on the distribution of the second dose of the Gd-liposome were evaluated.
Results. Results indicated that the tissue distribution of the second injection of the Gd-micelle at a dose of 33, 5, or 2μmol Gd/kg was not affected by the first injection of the Gd-micelle at different doses and time intervals or of the empty PEGylated liposome 7 days before. ABC of Gd-liposome at a dose of 2.3μmol Gd/kg (corresponding to 10 μmol lipids/kg) was observed when the empty PEGylated liposome or Gd-liposome, but not the Gd-micelle, $PEG_{500,000}$ or PEG-lipid, was pre-administered.
Conclusions. The hydrophobic core of the micelle or lipid bilayer of PEGylated liposome has a major effect on this phenomenon. These studies have significant implications for the evaluation of PEG-poly (L-lysine)-based micellar formulation of Gd-based contrast agents.

KEY WORDS: accelerated blood clearance; gadolinium; PEGylated liposome; polyethylene glycol (PEG); polymeric micelle.

INTRODUCTION

Long-circulating liposomes with surface-modified polyethyleneglycol (PEG) are often used as carriers of therapeutic agents, since they avoid capture by the reticuloendothelial system (RES) and can extend the systemic circulation time of agents, thereby improving drug delivery (1,2). It was hypothesized that PEG on the surface of liposomes forms a water shell, resulting in decreased adsorption of opsonins and subsequent phagocytosis by cells of the RES (3,4). However, PEGylated liposomes are known to lose their long-circulating property with multiple dosing. Recently, it has been reported that the first dose of PEGylated liposomes injected intravenously caused a loss of the long-circulating property and extensive accumulation in the liver at the second dose injected several days later in mice, rats, rabbit, and rhesus monkeys (5–11), a phenomenon known as accelerated blood clearance (ABC). Besides PEGylated liposomes, other nanocarriers, such as nanoparticles containing PEG, also produced this phenomenon (12). Therefore, ABC would have a significant impact on the application of long-circulating liposomes and nanoparticles with multiple administrations. In clinical applications of liposomal carriers, Gabizon *et al.* reported a reduced clearance of doxorubicin-containing PEGylated liposome in the repeated injections. This opposite behavior to the ABC phenomenon resulted from toxic activity of the encapsulated doxorubicin against the RES (13). Presently, the ABC phenomenon is not a problem in a cancer chemotherapy by the use of a PEG-liposomal carrier, whereas the ABC phenomenon in human clinics must be important for less toxic drug or gene delivery applications of the PEGylated liposomes.

To date, studies of ABC have focused mainly on PEGylated liposomes. Many factors can affect the extent to which ABC is induced by PEGylated liposomes. First of all, the dose of lipid plays an important role, with ABC enhanced at lower concentrations of lipid (6,7,12). Second, ABC occurs in a time-dependent manner (5,7). The time interval between the first and second doses is a key factor. Third, when the amount of PEGylated lipid in the first injection was ≤5 mol%, the second dose of PEGylated liposomes was eliminated more quickly from plasma than liposomes containing >10 mol% PEGylated lipid injected as a first dose (7,8). In addition, the ABC phenomenon

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was reported to be independent of liposomal size, surface charge, and PEG molecular weight (5,7,8).

During the past decade, polymeric micelles, supramolecular assemblies of block copolymers, have demonstrated their utility in drug delivery systems and are currently recognized as promising nanocarriers for enhancing the efficacy of drugs and genes (14–16). Since ABC has a considerable impact on the multiple drug administration, it is necessary to study whether the phenomenon is induced by repeated injections of polymeric micelles. Gadolinium (Gd)-based contrast agents are widely used in magnetic resonance imaging (MRI) to improve the conspicuity of lesions or visualization of blood vessels (17). However, these agents are rapidly cleared from the circulation. To overcome this problem, nanocarriers, such as liposomes and polymeric micelles, are used to encapsulate the agents so as to prolong their circulation and allow them to accumulate in tumors for diagnosis (18–20). If polymeric micelles containing a diagnostic agent cause the ABC phenomenon, then circulation time will be reduced after a second dose and the accuracy of the diagnosis will be affected. Furthermore, polymeric micelles containing MRI agents or drugs administered during diagnosis and treatment will lose some of their drug efficacy because of the accelerated clearance. Hence, it is of great importance to know whether the ABC phenomenon can be induced by polymeric micelles or not. Recently, the accelerated clearance of [^{3}H]-labeled PEGylated liposomes was observed in mice pre-administered with an empty polymeric micelle composed of poly (ethylene glycol)-*b*-poly (β-benzyl L-aspartate) (PEG-PBLA) 50 nm in diameter (16).

In this study, we first investigated whether the ABC effect was caused by repeated injections of a polymeric micelle encapsulating Gd-DOTA (Gd-micelle) and of a PEGylated liposome encapsulating Gd-DTPA (Gd-liposome) as a positive control. Concentrations of Gd ions were measured for this investigation. Furthermore, we examined the effect of a PEG homopolymer on the tissue distribution of Gd-liposomes.

MATERIALS AND METHODS

Materials

Magnevist® (Gd-DTPA) was purchased from Bayer Schering Pharma (Berlin, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE), hydrogenated soy bean phosphatidylcholine (HSPC), and egg phosphatidylcholine (EPC) were purchased from the NOF Corporation (Tokyo, Japan). Cholesterol and polyethylene glycol 500,000 (PEG$_{500,000}$) were of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

Animals

Four-week-old female ddY mice were purchased from Sankyo Lab Service Corp. (Tokyo, Japan). All care and handling of animals were performed with the approval of the Animal and Ethics Review Committee of Hoshi University and of Principles of Laboratory Animal Care (NIH #publication 85-23, revised in 1985).

Preparation of the Gd-micelle

Synthesis of a chelate moiety-binding block copolymer was performed as reported in our previous paper (19). Briefly, a poly(ethylene glycol)-*b*-poly(L-lysine) block copolymer (PEG-P(Lys)) was prepared through acid hydrolysis of a poly(ethylene glycol)-*b*-poly[ε-(benzyloxycarbonyl)-L-lysine] (PEG-P(Lys(Z)) block copolymer (Fig. 1). We synthesized PEG-P(Lys(Z) with polymerization of a Lys(Z) *N*-carboxy anhydride monomer from PEG-NH$_2$ (molecular weight of PEG-NH$_2$ = 5,200). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide ester) was fully conjugated to lysine residues of PEG-P(Lys).

The composition of PEG-P(Lys-DOTA) was determined by means of ^{1}H-NMR spectroscopy in D_2O under alkali conditions (pH>10). $GdCl_3 \cdot 6H_2O$ was added to PEG-P(Lys-DOTA) at pH 6.0 to 6.5 for 3 hr at 50°C. Gd content was determined using inductively coupled plasma (ICP) (SPS7800, SII Nano Technology Inc., Tokyo, Japan). We obtained the block copolymer as PEG-P(Lys-DOTA-Gd) (Gd content = 7.7 wt%, the number average of Gd is 8.2). The block copolymer formed a polymeric micelle spontaneously in an aqueous solution (Gd-micelle). The size and zeta-potential of the Gd-micelle diluted with saline for three independent preparations was 84.5±6.0 nm and −1.70±0.80 mV, respectively, at 25°C as determined by dynamic light scattering (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan).

Preparation of the Empty Liposome and Gd-liposome

First, an empty liposome, which induced the ABC phenomenon, was prepared by the lipid film hydration method as described previously (21). Briefly, a mixture of HSPC, cholesterol, and mPEG$_{2000}$-DSPE in a molar ratio of 1.85:1.0:0.15 was dissolved in chloroform. The solution was

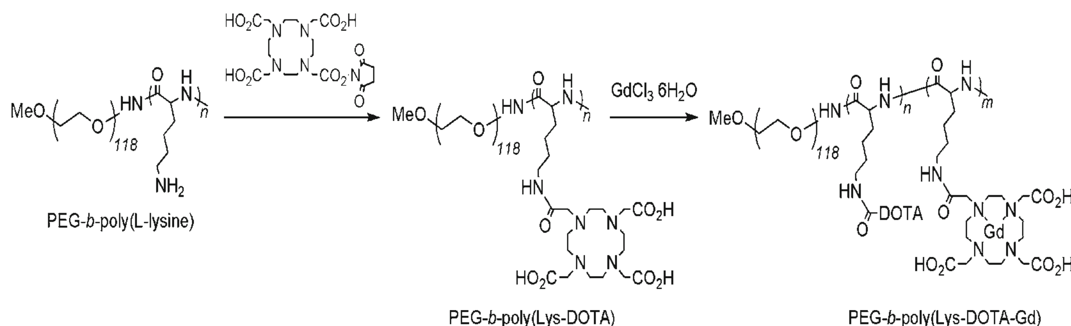

Fig. 1. Synthesis of PEG-P(Lys-DOTA-Gd).

evaporated dry to form the lipid film. Then, the liposome was produced by hydration of the lipid film with saline, followed by size reduction with sonication. The size and zeta-potential of the liposome diluted with saline were 178.5 nm and −22.1 mV, respectively.

Two kinds of Gd-liposomes were prepared because Gd-DTPA content was dependent on the preparation methods. One kind of Gd-liposome was prepared by an ethanol injection method (GdL-E). In brief, a mixture of EPC, cholesterol, and $mPEG_{2000}$-DSPE in a molar ratio of 2.15:0.88:0.15 was dissolved in ethanol and then hydrated with Gd-DTPA at 50°C. The resulting liposomes were sonicated for 10 min, then subjected to exhaustive dialysis against phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) with a dialysis membrane having a 2,000 molecular-weight cutoff for 24 hr. The size and zeta-potential of the liposome were 150.1±18.8 nm and −0.94±6.78 mV, respectively, for three independent preparations. As a control of GdL-E, an empty liposome not including Gd-DTPA (empty GdL-E) was prepared by the same method as the Gd-liposome (GdL-E), except that saline was used to hydrate the ethanol solution of lipid. The particle size of empty GdL-E was 139.5 nm. Another kind of Gd-liposome was prepared by reverse phase evaporation (GdL-R) to encapsulate a larger amount of Gd-DTPA. The lipid was the same as GdL-E described above and dissolved in 4 mL of chloroform and 2 mL of diethyl ether. Gd-DTPA was added to the lipid solution. The mixture was sonicated to form an emulsion, which was evaporated to produce the liposome. Finally, the resulting liposome was sized at 60°C on an extruder (Avanti Polar Lipids, Inc., AL, USA) with three passes through a 0.4 μm Nuclepore membrane (Waterman, Maidstone, UK) and five passes through a 0.2 μm Nuclepore membrane, followed by exhaustive dialysis as described above. The particle size and zeta-potential of the liposome were 140.9± 13.5 nm and −2.52±5.18 mV, respectively, for three independent preparations. The phospholipid concentration of the liposome including HSPC or EPC was measured with the Phospholipids C-test Wako (Wako Pure Chemical Industries, Ltd.). GdL-E contained 2.26 μmol Gd per 10 μmol lipids, and GdL-R contained 2.29 μmol Gd per 5 μmol lipids.

Release Studies of Gd-micelle and Gd-liposomes

The release of Gd−DTPA from Gd−liposome (GdL−E or GdL−R) and Gd from Gd−micelle was evaluated by dialysis method using a Spectrapor 6 tubing with molecular weight cut-off of 1,000 Da (Spectrum Laboratories Inc., Tokyo, Japan). Briefly, the sample of Gd-micelle containing 1.2 mM Gd and Gd-liposomes of GdL-E containing 0.96 mM Gd-DTPA or GdL-R containing 0.96 mM Gd-DTPA (1 mL) were dialyzed against PBS (pH 7.4, 200 mL) at 37°C. At the indicated time points (10 min, 1, 3, 6, 24 h), 1 mL aliquots of the medium were withdrawn, and the same volume of fresh medium was added. The Gd concentration was analyzed by ICP. The accumulative release of Gd or Gd-DTPA released from the Gd-micelle or Gd-liposome, respectively was expressed as a percentage of the released Gd or Gd-DTPA and plotted as a function of time.

Pharmacokinetics and Tissue Distribution of the Gd-micelle and Gd-liposome

For pharmacokinetics study, the mice were intravenously injected with the Gd-micelle at a dose of 33 μmol Gd/kg (67.3 mg polymer/kg) or the Gd-liposomes including GdL-E at a dose of 6.75 μmol Gd/kg and 10 μmol lipids/kg and GdL-R at 2.65 μmol Gd/kg and 5 μmol lipids/kg. About 30 to 100 μL of blood were taken from a tail vein with a quantitative capillary at 10 min, 1 h, 3 h, 6 h, and 24 h after the injection. The Gd-micelle or the Gd-liposome was injected into a lower part of a tail vein, and blood sample was taken at a certain time point described above from an upper part of the tail vein at the other side of the injected vein. Therefore, this experiment was free from the sample pollution problem. The blood samples were added to saline and centrifuged at 3,000 rpm for 15 min, and the supernatant was used to measure Gd content by ICP. The elimination half-life ($T_{1/2}$) was calculated based on a single compartment model. For the tissue distribution of Gd-micelles and Gd-liposomes study, the second dose of Gd-micelles or Gd-liposomes was injected intravenously through the tail vein at a certain time interval after the first injection. Samples of blood were taken from the hepatic portal vein 6 h after the second injection, and tissues of liver, spleen, and kidney were removed at the same time. The plasma and blood volume were calculated as 0.0488 mL/g body weight for plasma and 0.0778 mL/g body weight for blood, respectively (19).

Measurement of Gd Content

For the quantitative determination of Gd content, blood samples were centrifuged at 3,000 rpm for 15 min, and then plasma was taken out and diluted with 0.1% HNO_3 for ICP. Tissue samples of the liver, spleen, and kidney were digested with a mixture of 98% H_2SO_4 and 62% HNO_3 (1:2, *v*:*v*) and then subjected to ICP.

Statistical Analysis

The statistical analysis was performed with the Dunnett's multiple comparison test. The level of significance was set at $p<0.05$ or $p<0.01$.

RESULTS

Release Behavior of Gd-micelle and Gd-liposomes

Gd or Gd-DTPA release behavior from Gd-micelle or Gd-liposomes was studied by the dialysis method. As shown in Fig. 2, only 0.2% of Gd leaked from the Gd-micelle at 37°C in PBS (pH 7.4) for 24 h. On the other hand, 4.8% of entrapped Gd-DTPA leaked from the Gd-liposome prepared by reverse phase evaporation method (GdL-R) and 22.4% for 24 h from the Gd-liposome prepared by ethanol injection method (GdL-E). Hence, it is obvious that Gd-micelle has hardly release behavior of Gd, and GdL-R showed much slower release than GdL-E. The results indicated that the leakage of Gd or Gd-DTPA from nanocarriers was greatly affected by the preparation methods.

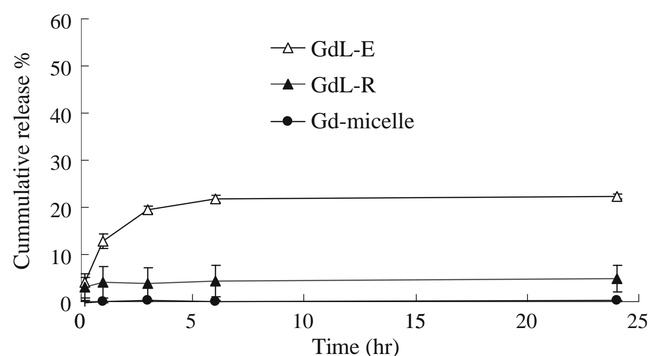

Fig. 2. Release profiles of Gd from Gd-micelle or Gd-DTPA from Gd-liposomes prepared by ethanol injection method (GdL-E) and reverse phase evaporation method (GdL-R) in PBS (pH 7.4) at 37°C. Data represent mean±S.D. (n=3).

Pharmacokinetics of the Gd-micelle and Gd-liposome

As shown in Fig. 3, at 10 min after the intravenous injection, 33.3% of the injected dose was found in blood for the Gd-micelle, and 40.0% and 50.3% for the Gd-liposome of GdL-E and GdL-R, respectively. At a dose of 33 μmol Gd/kg, the $T_{1/2}$ of the Gd-micelle was 10.2±3.9 h. Besides, the $T_{1/2}$ of GdL-E at a dose of 6.75 μmol Gd/kg and GdL-R at a dose of 2.65 μmol Gd/kg were 5.9±0.5 h and 6.0±1.0 h, respectively. In a previous study, we showed that Gd-DTPA was very rapidly cleared from the bloodstream with a minute's order half-life (19). Therefore, the detected Gd in blood is considered to be Gd-DTPA encapsulated in the liposome in a quantitative manner for measurements 6 h post intravenous injection. On the other hand, the main purpose of this study is the ABC phenomenon of a polymeric micelle MRI contrast agent, and PEGylated liposome is used as a positive control for the ABC phenomenon. Therefore, detection of liposome with Gd measurements is appropriate for the present purpose.

Effect of the First Dose on the Distribution of the Gd-micelle

The effects of the first dose on the distribution of the Gd-micelle injected a second time were evaluated. When the second dose of Gd-micelle was fixed at 33 μmol/kg, there was no significant difference of percent injected doses in plasma, kidney, and spleen between various first doses of the Gd-micelle from 0 to 100 μmol/kg (Fig. 4A). A dose of 100 μmol Gd/kg is the clinical dose of Gd-DTPA (17). Interestingly, the distribution of Gd-micelles in plasma, kidney, spleen and liver with the first injection of the empty liposome was similar to that with the first injection of saline. For the liver, the percent injected dose after a first dose of 33 μmol/kg and 100 μmol/kg was significantly higher than in the control saline group, possibly due to the incomplete elimination of the first dose of the Gd-micelle in liver at day 7 because of high doses of polymeric micelles (67.3 mg ~ 203.9 mg polymer/kg). The dose of 2 μmol Gd/kg of the Gd-micelle was the minimum at which Gd was detectable by means of ICP 6 h after injection. As shown in Fig. 4B, when the second dose of the Gd-micelle was decreased to 5 μmol/kg and 2 μmol/kg, the distribution was similar to that of 33 μmol/kg (Fig. 4A). Hence, the results showed that the tissue distribution of the Gd-micelle at the second dose of 33, 5, or 2 μmol/kg was not affected significantly except in liver by pre-administration of the Gd-micelle or the empty liposome. Although Gd in the first dose may interfere with the Gd accumulation in liver following the second dose injection, Gd-micelle as the first dose for micelle-forming properties are

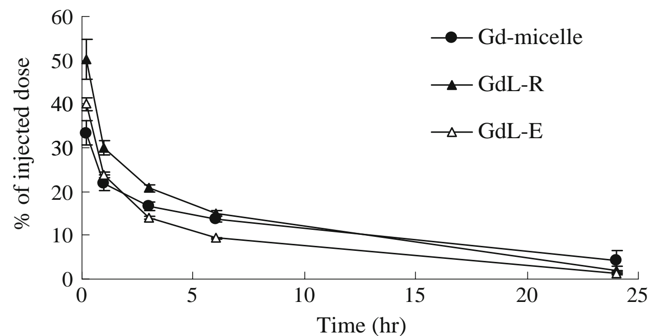

Fig. 3. Plasma elimination profiles of Gd following a single intravenous injection of Gd-micelle at a dose of 33 μmol Gd/kg and Gd-liposome including Gd-liposome prepared by ethanol injection method (GdL-E) at a dose of 6.75 μmol Gd/kg and Gd-liposome prepared by reverse phase evaporation method (GdL-R) at a dose of 2.65 μmol Gd/kg. Data represent mean±S.D. (n=3–4).

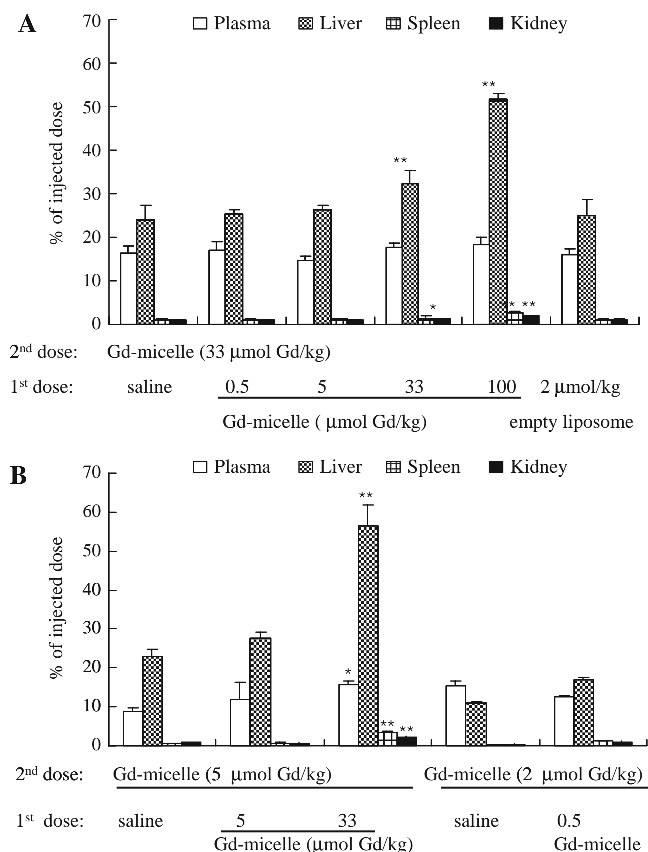

Fig. 4. Effect of the first dose on the tissue distribution of Gd-micelle. The second dose of Gd-micelle with 33 μmol/kg (**A**) or 5 μmol/kg or 2 μmol/kg (**B**) was intravenously injected at day 7 after the first injection of 0.5, 5, 33, 100 μmol/kg of Gd-micelle or the empty liposome at a dose of 2 μmol lipid/kg. Tissues including blood, liver, spleen, and kidney were taken out 6 h after the second injection of Gd-micelle. Data represent mean±S.D. (n=3, 6). P values apply to differences between the saline group and Gd-micelle or liposome treated group. *$p<0.05$, **$p<0.01$.

Gd-content-dependent, and Gd-free polymeric micelle is different from the Gd-containing micelle in size and micelle forming characteristics.

Effect of Time Interval Between the Two Injections on the Distribution of the Gd-micelle

Since it was reported that the ABC effect was maximized when the interval between the two injections of liposome was 10 days in mice (7,12), we changed the time interval for the injection of Gd-micelle at a dose of 33 μmol/kg from 3 days to 10 days. No significant difference in plasma Gd levels (15 ~ 18% dose) was observed between the control group and the groups with different time intervals 6 h after the second injection (Fig. 5). The control group was given the Gd-micelle at 33 μmol/kg after a first injection of saline. The Gd% of injected dose in the liver was much higher at day 3 after the second injection than that on other days, probably due to the incomplete elimination of the first dose of the Gd-micelle.

Effect of the First Dose on the Distribution of the Gd-liposome

Since a lower dose of lipid in the first injection results in a more significant ABC, the effects of dose were investigated. The first dose of the Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), or GdL-E (2 μmol lipids/kg corresponding to 0.45 μmol Gd/kg) was given with a second dose of GdL-E at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg. As shown in Fig. 6, the first injection of the Gd-micelle resulted in a similar percentage of the injected dose of the Gd-liposome in plasma, liver, spleen, and kidney in comparison with the saline group. On the other hand, after the second injection of GdL-E, the Gd concentrations in plasma and kidney were too low to be detected, with the first injection of the empty GdL-E and the GdL-E. At that time, the %dose in the liver significantly increased, but that in spleen significantly decreased as compared to saline ($p<0.05$).

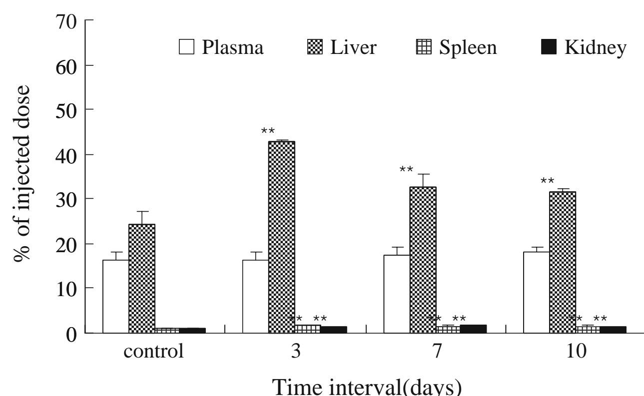

Fig. 5. Effect of the time intervals on the tissue distribution of Gd-micelle. The second dose of Gd-micelle at 33 μmol/kg was intravenously injected at day 3, day 7, or day 10 after the first injection of the same micelle at 33 μmol/kg. The control group was referred to the second dose of Gd-micelle at a dose of 33 μmol/kg with the first injection of saline. Tissues including blood, liver, spleen, and kidney were taken out at 6 h after the second injection of Gd-micelle. Data represent mean±S.D. (n=3). P values apply to differences between the control group and treated group. *$p<0.05$, **$p<0.01$.

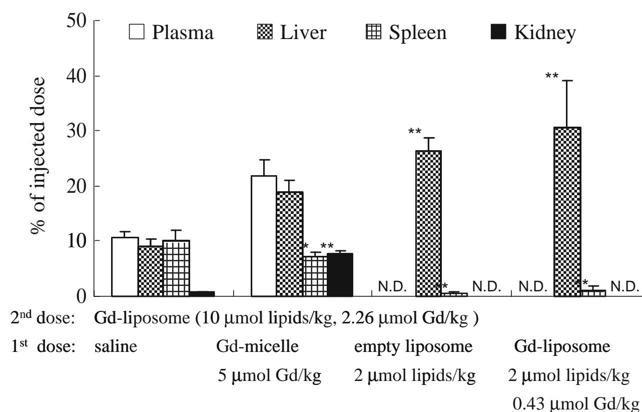

Fig. 6. Effect of the first dose on the tissue distribution of Gd-liposome (GdL-E). The second dose of GdL-E with 10 μmol lipids/kg and 2.26 μmol Gd/kg was intravenously injected at day 7 after the first injection of Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), and GdL-E (2 μmol lipids/kg and 0.43 μmol Gd/kg). Tissues of blood, liver, spleen, and kidney were removed 6 h after the second injection of GdL-E liposome. Data represent mean±S.D. (n=3). P values apply to differences between the saline group and Gd-micelle or liposome treated group. *$p<0.05$, **$p<0.01$. N.D. The Gd concentration was too low to be detected by ICP.

Therefore, the data herein show that the accelerated clearance of Gd-liposome at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg was induced by both the Gd-liposome and empty liposome, but not by the Gd-micelle. This finding indicates that Gd ions at the first dose of 0.45 μmol/kg did not affect the induction of ABC caused by liposomes.

Effect of PEG on the Distribution of Gd-liposomes

Next, the effect of injecting a PEG homopolymer and PEG_{2000}-DSPE on the distribution of Gd-liposomes was examined. Since the encapsulation efficiency of Gd was low with the ethanol injection method, we prepared another Gd-liposome by the reverse phase evaporation method (GdL-R). The tissue distribution of GdL-R at 6 h after injection at a dose of 5 μmol lipids/kg was not significantly influenced by the pre-administration of 50 mg/kg PEG $_{500,000}$, 0.2 mg/kg PEG $_{500,000}$, or 0.3 mg/kg PEG_{2000}-DSPE 7 days before (Fig. 7). The dose of 0.2 mg/kg PEG $_{500,000}$ and 0.3 mg/kg PEG_{2000}-DSPE with the concentration of 0.04 mg/ml is similar to that of the 5 mol% PEGylated liposome (0.3 mg/kg PEG_{2000}-DSPE), which could produce the ABC phenomenon (Fig. 6). Hence, the first injection of $PEG_{500,000}$ saline or PEG_{2000}-DSPE saline failed to cause the ABC phenomenon after the second administration of Gd-liposome. Hence, only injections of PEG macromolecules did not induce the ABC effect.

DISCUSSION

In the present study, the influence of dose on the tissue distribution of Gd-micelles after repeated administrations was investigated. Many studies have found that a lower dose of lipid in liposomes or nanoparticles results in a greater ABC effect (6–8,12), and the magnitude of the ABC phenomenon reached a maximum when the time interval between two

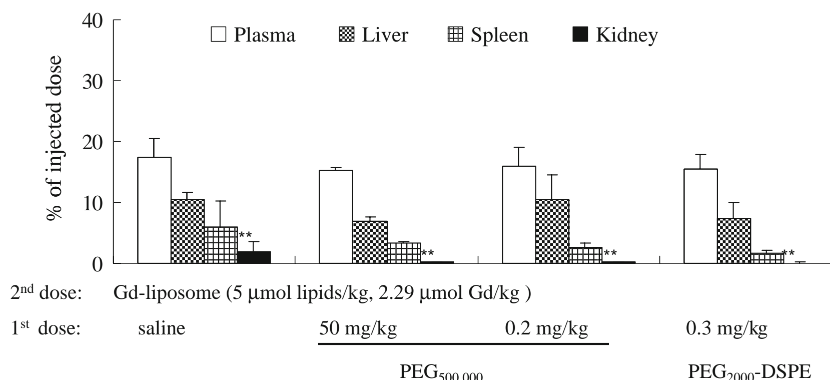

Fig. 7. Effect of $PEG_{500,000}$ and PEG_{2000}-DSPE on the tissue distribution of Gd-liposome (GdL-R). The second dose of Gd-liposome with 5 μmol lipids/kg and 2.29 μmol Gd/kg was intravenously injected at day 7 after the first injection of $PEG_{500,000}$ saline at a dose of 50 mg/kg or 0.2 mg/kg, and PEG_{2000}-DSPE saline at 0.3 mg/kg. The control group of GdL-R was injected at a dose of 10 μmol lipids/kg with the first injection of saline. Tissues of blood, liver, spleen, and kidney were removed at 6 h after the second injection of GdL-R. Data represent mean±S.D. (n=3–5). P values apply to differences between the saline group and $PEG_{500,000}$ or PEG_{2000}-DSPE treated group. *$p<0.05$, **$p<0.01$.

injections was 5–7 days in rats (5) and 10 days in mice (7,12). Hence, we investigated the distribution of the Gd-micelle at various doses and an interval of 3, 7, or 10 days between injections. We found that repeated injections of the Gd-micelle, even with the second dose reduced to 2 μmol Gd/kg (corresponding to 4 mg polymer/kg) and at different time intervals at a dose of 33 μmol Gd/kg (corresponding to 67.3 mg polymer/kg), did not result in an accelerated clearance. ABC of the second injection of the Gd-liposome was induced by the first injection of both the Gd-liposome and the empty PEGylated liposome, but not by the first injection of the Gd-micelle (Fig. 6), Gd-DTPA encapsulated in liposomes would not affect the ABC phenomenon. Therefore, our observation that the ABC phenomenon did not occur with the Gd-micelle is important as it means that injections of the Gd-micelle will not change the biodistribution of a second administration of diagnostic or therapeutic agents.

For Gd-micelle, Gd was chelated to the micelle and thus existed in the form of micelle as shown in Fig. 1, which was consistent with the release results in Fig. 2 that Gd did not leak from the Gd-micelle in PBS (pH 7.4). Gd concentration in plasma, therefore, will reflect the pharmacokinetic behavior of the Gd-micelle. For Gd-liposomes, although the Gd concentration in plasma contained both the leaked Gd-DTPA from the Gd-liposome and the encapsulated Gd-DTPA in the Gd-liposome, the leaked-free Gd-DTPA is reported to be very rapidly cleared from the bloodstream with a minute's order half-life (19), and thus the detected Gd in blood is considered to be only the Gd-DTPA encapsulated in the liposome 6 h after intravenous injection in this study. Compared to GdL-R, the leakage of Gd-DTPA from GdL-E was faster, resulting in the lower Gd concentration (the encapsulated Gd) of GdL-E in blood in Fig. 3. Most importantly, the purpose of this study is to investigate if the distribution for the second dose of the Gd-liposomes or Gd-micelle was affected after pre-administered with the first dose or not. Therefore, the leakage of Gd-DTPA from the Gd-liposomes will not influence this study. In addition, many studies demonstrated that ABC phenomenon for empty liposome was observed determined by $[H^3]$-labelled or ^{99m}Tc-labelled method (5–9).

It is believed that macrophages in the RES play an important role in ABC, and liposomes were mainly located in Kupffer cells after a second injection (5,8).When hepatosplenic macrophages were depleted, no enhanced clearance of liposomes was observed (6). The induction of ABC with liposomes could be attributable to a 150 kDa serum factor (5), anti-PEG IgM (9,11,12,22,23), anti-PEG antibody (10), or anti-PEG IgG antibody (24).

Whereas the mechanism of the immune response on repeated injections of liposomes has not been fully elucidated yet, the enhanced clearance effect can still be divided into two phases: the induction phase following the first injection and the effectuation phase following the second injection (6). According to this theory, there are two very important factors: one is the biological material (e.g. antibody) produced in the induction phase, the other is the recognition of the antibody by the second dose. For the effectuation phase, it was reported that the ABC phenomenon was induced by the second dose of a PEGylated liposome, but not of a liposome lacking a PEG-coating (23). This indicates that PEG is essential for the nanocarrier to recognize the antibody in the effectuation phase. In this study, the ABC phenomenon was not observed after repeated injections of the Gd-micelle at different doses and time intervals. This ABC failure of Gd-micelle may be caused by the failure for the production of biological material in the induction phase (data not shown) or/and for the recognization by the antibody in the effectuation phase. Even if the first injection was of empty liposome, the second injection of the Gd-micelle did not produce the ABC phenomenon either. This suggests that the antibody produced by the empty liposome in the induction phase is not recognized by the PEG moiety of the Gd-micelle. Therefore, not only PEG, but also other factors such as structure and hydrophobic character affect recognition.

For the induction phase, the ABC phenomenon was not observed when the amount of PEGylated lipid of liposomes in the first injection was more than 10 mol% (7,8). We have previously reported the accelerated clearance of $[^3H]$-labeled PEGylated liposomes in mice pre-administered empty PEG-PBLA polymeric micelles (16). Furthermore, repeated

injections of PEG-PLA nanoparticles also produced the ABC phenomenon (12). Hence, the structure and component of nanocarriers has a considerable impact on the induction phase of ABC. From a structural perspective, the Gd-micelle formed through ionic interactions; therefore, it does not have any hydrophobic part (Fig. 1). In contrast, the PEG-PBLA micelle is composed of both a hydrophilic part, PEG, and a hydrophobic part, PBLA. Similarly, PEGylated liposomes possess a hydrophilic PEG chain and a hydrophobic bilayer membrane. The immunogenicity of an antigen can be affected by factors such as the physical and chemical properties of the antigen, its dose, and so on (25). The reasons why the Gd-micelle evaded the ABC phenomenon have not yet been elucidated at the present stage. The absence of a hydrophobic part may be a key for this elucidation because the other ABC-phenomenon-positive PEG-based carrier systems possess hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. We are currently investigating the ABC phenomenon induced by other kinds of polymeric micelles and nanoparticles. It is hoped that these experiments will provide more evidence for the mechanism of the ABC phenomenon.

CONCLUSIONS

The Gd-micelle did not induce ABC following its pre-administration at various doses and time intervals. In contrast, the Gd-liposome induced the phenomenon when it or an empty PEGylated liposome, but not the $PEG_{500,000}$ macromolecule or PEG_{2000}-DSPE, was pre-administered. ABC-phenomenon-positive PEG-based carrier systems possess a hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. The absence of a hydrophobic part of Gd-micelle may be a key factor for not producing the ABC phenomenon.

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