International Journal of Pharmaceutics 392 (2010) 218–223

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

T cell-independent B cell response is responsible for ABC phenomenon induced by repeated injection of PEGylated liposomes

Hiroyuki Koide[a], Tomohiro Asai[a], Kentaro Hatanaka[a], Shuji Akai[b], Takayuki Ishii[a], Eriya Kenjo[a], Tatsuhiro Ishida[c], Hiroshi Kiwada[c], Hideo Tsukada[d], Naoto Oku[a,*]

[a] Department of Medical Biochemistry and Global COE Program, Graduate School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[b] Department of Synthetic Organic Chemistry, Graduate School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan
[c] Department of Pharmacokinetics and Biopharmaceutics, Institute of Health Biosciences, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan
[d] Central Research Laboratory, Hamamatsu Photonics K.K., Hamamatsu, Shizuoka, Japan

ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form 8 February 2010
Accepted 8 March 2010
Available online 21 March 2010

Keywords:
Polyethylene glycol
Liposome
Accelerated blood clearance
Thymus-independent type 2 antigen
Nanocarriers

ABSTRACT

Repeated injection of polyethyleneglycol-modified (PEGylated) liposomes causes a rapid clearance of them from the bloodstream, this phenomenon is called accelerated blood clearance (ABC). In the present study, we focused on the immune system responsible for the ABC phenomenon. PEGylated liposomes were preadministered to BALB/c mice and [^{3}H]-labeled ones were then administered to them 3 days after the preadministration. Consistent with our previous results, the preadministration with PEGylated liposomes triggered the rapid clearance of [^{3}H]-labeled PEGylated liposomes from the bloodstream, but that with PEGylated liposomes encapsulating doxorubicin (Dox) did not. In addition, we found that the ABC phenomenon was observed when a mixture of free Dox and PEGylated liposomes was preadministered. These data indicate that immune cells responsible for the ABC phenomenon might be selectively damaged by the Dox encapsulated in PEGylated liposomes. The ABC phenomenon was also observed in BALB/c *nu*/*nu* mice, but not in BALB/c SCID mice. The amount of anti-PEG IgM antibody induced by the stimulation with the PEGylated liposomes was significantly increased in the BALB/c *nu*/*nu* mice, but not in the BALB/c SCID ones. These data indicate that a T cell-independent B cell response would play a significant role in the ABC phenomenon. Furthermore, the present study suggests that PEGylated liposomes might be recognized by B cells as a thymus-independent type 2 (TI-2) antigen. The present study provides important information for the future development of liposomal medicines.

1. Introduction

PEGylated liposomes have been widely investigated as drug carriers and gene delivery systems. PEG forms a water shell on the liposomal surface and provides a steric barrier to the liposomes for avoiding interactions with plasma proteins, resulting in escape from trapping by the reticuloendothelial system (Lasic et al., 1991; Torchilin et al., 1994; Van Rooijen and Van Nieuwmegen, 1980). Therefore, PEGylated liposomes have the property of long circulation and are useful for drug delivery to tumors and inflamed sites, resulting in improving the therapeutic indices of encapsulated drugs (Allen, 1994). As a representative example of liposomal drugs, Doxil® has been used clinically. Doxil® is PEGylated liposomes encapsulating doxorubicin, which is used for reducing side effects of Dox such as cardiotoxicity and for enhancing its anticancer activity through enhanced permeability and retention (EPR) effect (Berry et al., 1998; Maeda et al., 2000; Muggia, 1999). On the other hand, we and others have found that a repeat injection of PEGylated liposomes into certain animals such as mice, rats and rhesus monkey triggers the rapid clearance of the second dose through their accumulation in the liver (Dams et al., 2000; Ishida et al., 2003a,b). This phenomenon, called as the accelerated blood clearance (ABC) phenomenon, is expected to have a considerable impact on the clinical use of liposomal formulations (Dams et al., 2000; Ishida et al., 2003a,b). A previous study of ours indicated that when rats were pretreated with a high dose (more than 5 μmol phospholipids/kg) of PEGylated liposomes, the induction of the ABC phenomenon was weakened. However, when rats were pretreated with a low concentration of them (1 μmol phospholipids/kg), the phenomenon was strongly induced (Ishida et al., 2006a,b,c). This phenomenon was widely observed even if the content of PEG lipid in liposomes or the length of the

Abbreviations: ABC phenomenon, accelerated blood clearance phenomenon; [^{3}H]-CHE, [^{3}H] cholesterylhexadecyl ether; MPEG-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]; MPS, mononuclear phagocyte system; PEGylated liposomes, polyethylene glycol-modified liposomes.

* Corresponding author. Tel.: +81 54 264 5701; fax: +81 54 264 5705.
E-mail address: oku@u-shizuoka-ken.ac.jp (N. Oku).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2010.03.022

PEG chain was varied. In fact, both methoxy(polyethyleneglycol)-2000-distearoylphosphatidylethanolamine ($mPEG_{2000}$-DSPE) and $mPEG_{5000}$-DSPE induced the phenomenon, and the concentration of $mPEG_{2000}$-DSPE in the first-dose PEGylated liposomes did not affect on the induction of the phenomenon (Ishida et al., 2005). In an earlier study, we also demonstrated that the spleen could play a key role in induction of the ABC phenomenon via secretion of anti-PEG IgM antibody by splenic B cells (Ishida et al., 2006a,b,c). Anti-PEG IgM antibody was gradually secreted by the administration of PEGylated liposomes and bound to the liposomes in the secondary injection, resulting in the rapid clearance of them from the bloodstream via complement activation (Ishida et al., 2005, 2007). Furthermore, the ABC phenomenon was triggered by preadministration with not only PEGylated liposomes but also polymeric micelles having PEG chains (Koide et al., 2008).

Our recent reports showed that the administration of Dox encapsulated in PEGylated liposomes (PEG–Dox) did not alter the pharmacokinetics of PEGylated liposomes injected as the test-dose (Ishida et al., 2006a,b,c; Laverman et al., 2001). In the present study, we firstly focused on the effect of Dox in the liposomes on the immune cells responsible for the ABC phenomenon. Then, we investigated the immune mechanism involved in this phenomenon.

2. Materials and methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), cholesterol (Cho), and distearoylphosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (mMPEG-DSPE) were kindly donated by Nippon Fine Chemical Co., Ltd. (Takasago, Hyogo, Japan). [^{3}H]cholesterylhexadecyl ether ([^{3}H]-CHE) was purchased from Amersham Pharmacia (Buckinghamshire, UK). All other reagents were analytical grade.

2.2. Animal

Five-week-old male BALB/c and BALB/c *nu*/*nu* mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Five-week-old male CB17/Icr-Prkdc^{scid}/CrlCrlj (BALB/c SCID) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were cared according to the animal facility guidelines of the University of Shizuoka.

2.3. Preparation of liposomes

PEGylated liposomes composed of DPPC and Cho with mPEG-DSPE (10:5:1 as a molar ratio) were prepared as described previously (Maeda et al., 2004). In brief, lipids dissolved in chloroform were evaporated to obtain a thin lipid film. Then, liposomes were formed by hydration with 10 mM phosphate-buffered 0.3 M sucrose solution and then sized by 5-times extrusion through a polycarbonate membrane filter with 100 nm pores (Nucleopore, Maidstone, UK). For a biodistribution study, a trace amount of [^{3}H]-CHE (74 kBq/mouse) was added to the initial chloroform solution. Dox-encapsulated liposomes were prepared by a modification of the remote loading method as described previously (Oku et al., 1994). The concentration of Dox was determined by its absorbance at 484 nm. The particle size of PEGylated liposomes was measured by use of a Zetasizer Nano ZS (MALVERN, Worcestershire, UK) after dilution of the liposomes with PBS, pH 7.4.

2.4. Biodistribution of PEGylated liposomes

Mice received an intravenous injection of PEGylated liposomes (2.0 μmol phospholipids/kg), PEG–Dox liposomes (1 mg/kg as a Dox dosage), a mixture of free Dox (1 mg/kg) and "empty" PEGylated liposomes or PBS. Three days later, [^{3}H]-labeled PEGylated liposomes (5.0 μmol phospholipids/kg) were administered to the mice via a tail vein. Twenty-four hours after the second administration, these mice were sacrificed under deep anesthesia for the collection of blood. Then, the blood was heparinized and separated by centrifugation (700 × *g*, 15 min, 4 °C) to obtain the plasma. After the mice had been bled from the carotid artery, their heart, lungs, liver, spleen, and kidneys were removed and weighed. The radioactivity in plasma and each organ was determined with a liquid scintillation counter (LSC-3100, Aloka, Tokyo, Japan). Tissue distribution data were presented as % dose per wet tissue. The total radioactivity in the plasma was calculated based on the average body weight of the mice, where the average plasma volume was assumed to be 4.27% of the body weight based on the data on total blood volume.

2.5. Detection of anti-PEG IgM antibody

Mice were intravenously injected with PEGylated liposomes (2.0 μmol phospholipids/kg), PEG–Dox liposomes (1 mg/kg as a Dox dosage), a mixture of free Dox (1 mg/kg) and "empty" PEGylated liposomes or PBS. Three days later, these mice were sacrificed and their blood was withdrawn. Serum was collected after centrifugation (700 × *g*, 15 min, 4 °C). To prepare the ELISA plates, 10 μg of mPEG-DSPE in 20 μL ethanol was added to 96-well plates (Nunc, Roskilde, Denmark). Then, the plates were air dried for 2 h to complete dryness and subsequently blocked with 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO) in PBS for 1 h. The diluted serum samples (100 μl) were added to the plates, incubated for 1 h, and washed 5 times with 1% FBS-PBS. Antibodies bound to mPEG-DSPE were detected with HRP-conjugated goat anti-mouse IgM antibody (Bethyl Laboratories, TX, USA). After incubation with the anti-IgM antibody for 1 h, each well was washed 5 times with 1% FBS-PBS. The coloration was initiated by the addition of *o*-phenylene diamine dihydrochloride (Sigma, St. Louis, MO, USA) that had been diluted with distilled water. After a 15-min incubation, the reaction was stopped by adding 100 μL of 2 M H_2SO_4, and the absorbance was recorded at 490 nm.

2.6. Synthesis of a positron emitter-labeled probe

The synthesis of 1-[^{18}F] fluoro-3,6-dioxatetracosane (SteP2) was prepared as described previously (Urakami et al., 2007). Briefly, [^{18}F] fluoride was produced with a cyclotron (HM-18, Sumitomo Heavy Industries, Tokyo, Japan) at Hamamatsu Photonics PET Center, and the labeled compound was synthesized from the precursor.

2.7. [^{18}F]-Labeling of PEGylated liposomes

[^{18}F]-Labeling of PEGylated liposomes were performed by a solid-phase transition (SophT) method (Urakami et al., 2007). About 100 MBq of [^{18}F]-SteP2 in ethanol solution was transferred to a glass test tube, and the solvent was removed completely at 90 °C with a flow of helium gas. PEGylated liposomes were added to the tube and incubated at 37 °C for 15 min with 5-s mixing by use of a vortex stirrer every 3 min. After the incubation, the PEGylated liposomes were centrifuged at 100,000 × *g* for 15 min (Beckman, Fullerton, CA, USA), and the supernatant was transferred to a new tube. Radioactivity of supernatant, precipitate, and original tube for labeling was measured with a curiemeter (IGC-3, Aloka, Tokyo, Japan) to calculate the labeling efficiency.

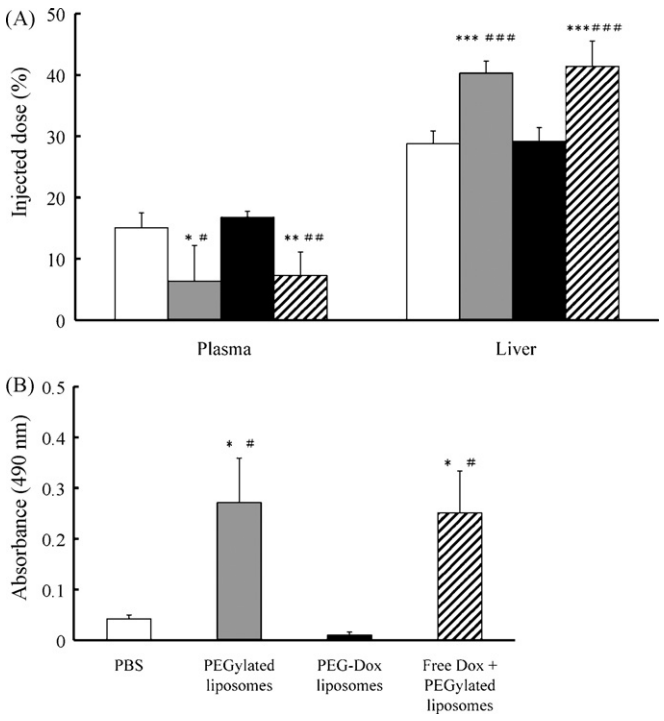

Fig. 1. Abolishment of the ABC phenomenon with Dox encapsulated in PEGylated liposomes. BALB/c mice were intravenously injected with PBS, PEGylated liposomes, PEG–Dox liposomes or a mixture of free Dox and "empty" PEGylated liposomes. (A) Biodistribution of the test-dose ^{3}H-labeled PEGylated liposomes: 3 days after the pretreatment, ^{3}H-labeled PEGylated liposomes were intravenously injected into these mice (5 μmol DPPC dosage/kg). Twenty-four hours after the second injection, the mice were sacrificed, and the radioactivity in the plasma and each organ (only liver data shown) was determined. Data ($n = 5$) are presented as a percentage of the injected dose per tissue and S.D. and (B) anti-PEG IgM in the serum collected at day 3 after the pretreatment. Each value represents the mean ± S.D. of 3 separate experiments. Data are presented for PBS (open bar), PEGylated liposomes (gray bar), PEG–Dox liposomes (closed bar), and a mixture of free Dox and PEGylated liposomes (hatched bar). Significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* PBS; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* PEG–Dox.

2.8. Imaging of [^{18}F]-labeled PEGylated liposomes by planar positron imaging system (PPIS)

Biodistribution of [^{18}F]-labeled PEGylated liposomes was determined with a positron planar imaging system (PPIS, Hamamatsu Photonics, Shizuoka, Japan). Mice anesthetized with chloral hydrate were positioned prone on an acrylic plate and placed between the 2 opposing detectors. An [^{18}F]-labeled sample at the dose of 2.5 MBq was intravenously injected into a mouse via a tail vein. The data were acquired with a 1-min time frame interval for 60 min, and 2 summation images were created every 30 min.

2.9. Statistics

Variance in a group was evaluated by using Student's *t*-test.

3. Results

3.1. Abolishment of ABC phenomenon by preadministration with Dox encapsulated in PEGylated liposomes

Mice were intravenously injected with PEGylated liposomes, PEG–Dox liposomes, a mixture of free Dox and "empty" PEGylated liposomes, or PBS for preconditioning. Three days later, these mice were administered PEGylated liposomes labeled with [^{3}H]-CHE as the test-dose. Fig. 1A shows the biodistribution of the test-dose PEGylated liposomes 24 h after the injection. Pretreatment with the PEG–Dox liposomes did not alter the plasma level or hepatic uptake of the test-dose compared with that with PBS. However, in case of pretreatment with the mixture of free Dox and "empty" PEGylated liposomes, the amount of test-dose significantly decreased in the plasma and significantly increased in the liver. The biodistribution pattern for this group was similar to that for the PEGylated liposome-injected group. The accumulation of the test-dose in other organs did not show significant differences among all groups tested (data not shown). Next, anti-PEG IgM antibody secretion was examined 3 days after the preconditioning (Fig. 1B). When either PEGylated liposomes or the mixture of free Dox and PEGylated liposomes were administered, production of anti-PEG IgM antibody was increased about 6-fold compared with the baseline level obtained for the PBS group. Whereas, when the mice were injected with the PEG–Dox liposomes, the production did not increase at all.

3.2. Imaging of biodistribution of PEGylated liposomes with PPIS

To assess the ABC phenomenon non-invasively, we next examined the change in the real-time distribution of PEGylated liposomes by use of PPIS. [^{18}F]-Labeled PEGylated liposomes were intravenously administered to the mice that had been pretreated with PEGylated liposomes, PEG–Dox liposomes or PBS 3 days before, and the biodistribution was imaged (Fig. 2). The biodistribution of [^{18}F]-labeled PEGylated liposomes was imaged for 60 min, and the images were integrated into 1–30 and 31–60 min composite images. In the 1–30 min image, weak [^{18}F] signals were observed in the lung, spleen, kidney and bladder, and strong signals were detected in the liver, particularly in the mice pretreated with PEGylated liposomes. In the 31–60 min image, these signals were reduced in the lung, spleen, kidney and liver in the groups pretreated with PBS or PEG–Dox, and were significantly increased in the bladder in both groups. However, relatively strong signals remained in the group pretreated with the PEGylated liposomes.

3.3. Induction of ABC phenomenon in BALB/c nu/nu mice

BALB/c *nu*/*nu* (T cell-deficient) mice were preadministered PBS, PEGylated liposomes or PEG–Dox ones to clarify the role of T cells in the induction of the ABC phenomenon. In the mice pretreated with PEGylated liposomes, the amount of PEGylated liposomes significantly decreased in the plasma and significantly increased in the liver. This indicates that the ABC phenomenon was induced in BALB/c *nu*/*nu* mice (Fig. 3A). Consistent with the data for BALB/c mice, the pretreatment with PEG–Dox liposomes did not alter the pharmacokinetics of the test-dose PEGylated liposomes in these BALB/c *nu*/*nu* mice. The anti-PEG IgM antibody production was significantly increased in the mice pretreated with the PEGylated but not PEG–Dox liposomes (Fig. 3B). All results obtained for the BALB/c *nu*/*nu* mice were similar to those obtained for the BALB/c ones.

3.4. Significant role of B cells in the ABC phenomenon

Since T cells did not seem to be involved in the induction of the ABC phenomenon, we next determined the function of B cells in this phenomenon by using SCID mice, which are known to be deficient in both T and B cells. The biodistribution of test-dose PEGylated liposomes in BALB/c SCID mice was determined. As a result, any preadministrations (PEGylated liposomes, PEG–Dox liposomes, or PBS) did not alter the pharmacokinetics of the test-dose PEGylated liposomes. These results indicate that the ABC phenomenon was not induced in BALB/c SCID mice and that B cells play an important role in the induction of the ABC phenomenon (Fig. 4A). In addition, anti-PEG IgM antibody was not detected in any of the BALB/c SCID groups (Fig. 4B).

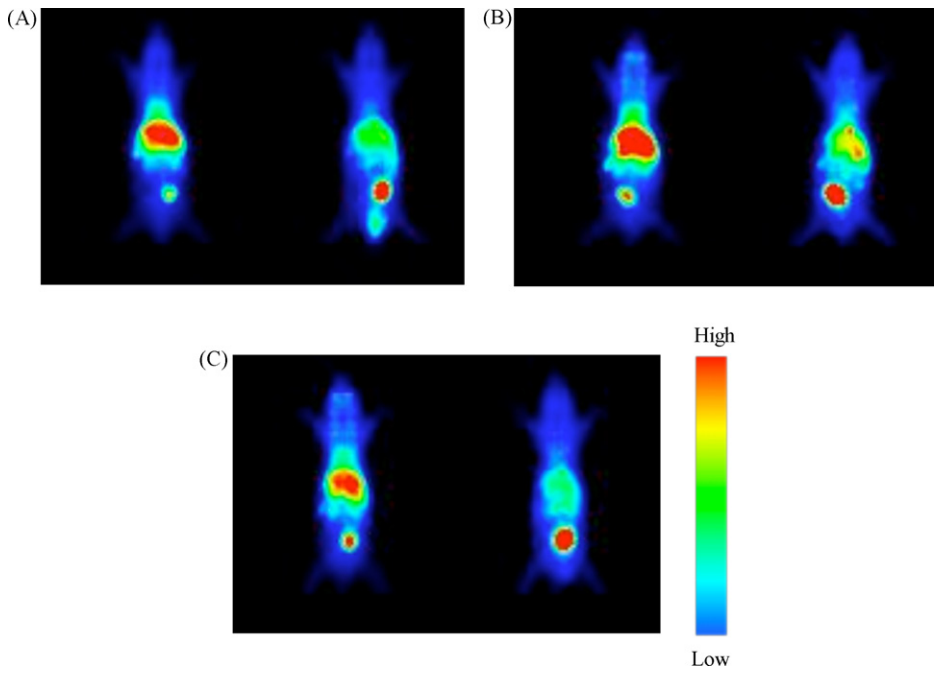

Fig. 2. Imaging of biodistribution of PEGylated liposomes with PPIS. Whole-body imaging of biodistribution of [^{18}F]-labeled PEGylated liposomes in BALB/c mice pretreated with PBS (A), PEGylated liposomes (B), or PEG–Dox liposomes (C) was performed by use of PPIS. Data were integrated into 1–30 (left image) or 31–60 min (right image) periods following the injection of [^{18}F]-labeled PEGylated liposomes.

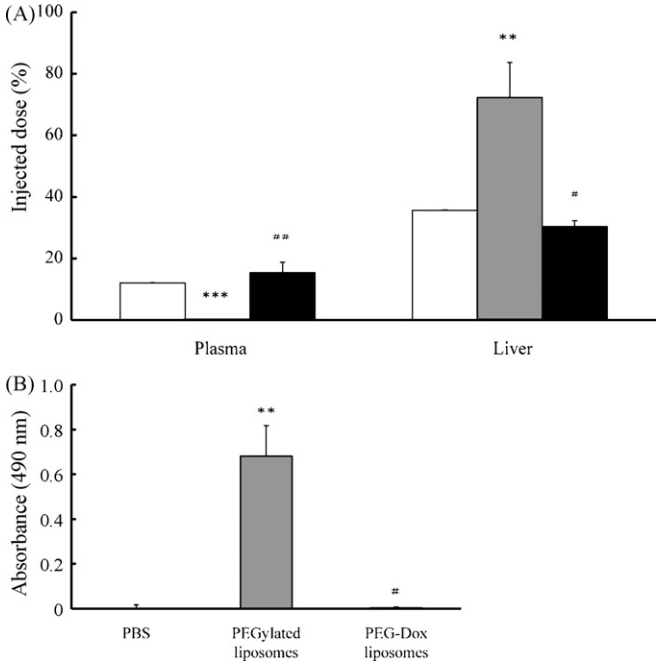

Fig. 3. Induction of the ABC phenomenon in BALB/c *nu*/*nu* mice. BALB/c *nu*/*nu* mice were intravenously injected with PBS, PEGylated liposomes or PEG–Dox liposomes. (A) Biodistribution of the test-dose ^{3}H-labeled PEGylated liposomes: 3 days after the pretreatment, ^{3}H-labeled PEGylated liposomes were intravenously injected into these mice (5 μmol DPPC dosage/kg). Twenty-four hours after the second injection, the mice were sacrificed, and the radioactivity in the plasma and each organ (only liver data shown) was determined. Data ($n = 5$) are presented as a percentage of the injected dose per tissue and S.D. and (B) anti-PEG IgM in the serum collected at day 3 after the pretreatment. Each value represents the mean ± S.D. of 3 separate experiments. Data are presented for PBS (open bar), PEGylated liposomes (gray bar), and PEG–Dox liposomes (closed bar). Significant differences: $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs*. PBS; $^{\#\#}p < 0.01$ and $^{\#}p < 0.001$ *vs*. PEGylated liposomes.

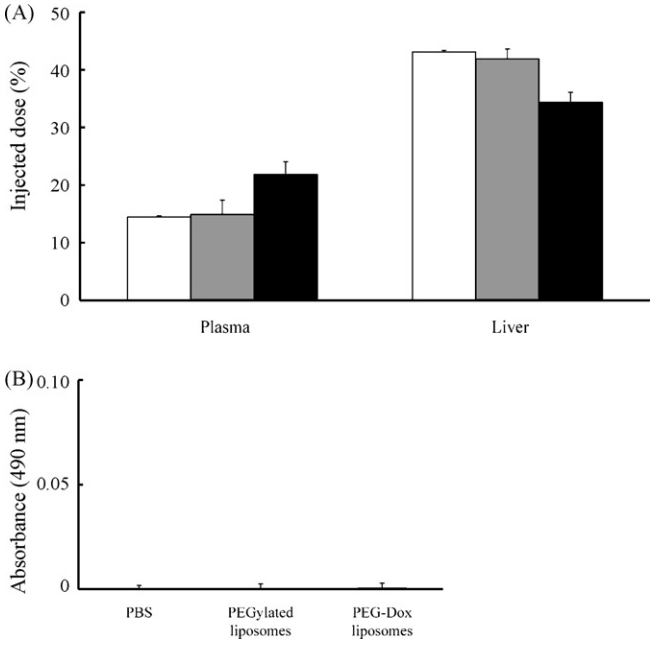

Fig. 4. No induction of the ABC phenomenon in BALB/c SCID mice. BALB/c SCID mice were intravenously injected with PBS, PEGylated liposomes or PEG–Dox liposomes. (A) Biodistribution of the test-dose ^{3}H-labeled PEGylated liposomes: 3 days after the pretreatment, ^{3}H-labeled PEGylated liposomes were administered via a tail vein (5 μmol DPPC dosage/kg). Twenty-four hours after the second injection, these mice were sacrificed; and the radioactivity in the plasma and each organ was then determined. Data ($n = 5$) are presented as a percentage of the injected dose per tissue and S.D. and (B) anti-PEG IgM in the serum collected at day 3 after the pretreatment. Each value represents the mean ± S.D. of 3 separate experiments. Data are presented for PBS (open bar), PEGylated liposomes (gray bar), and PEG–Dox (closed bar).

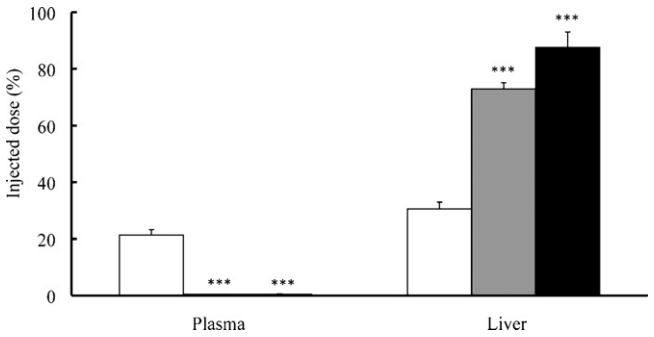

Fig. 5. Induction of the ABC phenomenon with non-PEGylated liposomes. BALB/c mice were intravenously injected with PBS, PEGylated liposomes, or non-PEGylated liposomes. Three days after pretreatment, ^{3}H-labeled PEGylated liposomes were administered via a tail vein. Twenty-four hours after the second injection, these mice were sacrificed; and the radioactivity in the plasma and each organ was then determined ($n = 5$). Data are presented as a percentage of the injected dose per tissue and S.D. Data are presented for PBS (open bar), PEGylated liposomes (gray bar), and non-PEGylated liposomes (closed bar), respectively. Significant differences: $^{***}p < 0.001$ *vs.* PBS.

3.5. Low specificity in the induction of the ABC phenomenon

We next focused on the specificity in the induction of the ABC phenomenon since T cell-independent B cell response against thymus-independent type 2 (TI-2) antigen might trigger this phenomenon. To elucidate the specificity in the induction of the ABC phenomenon, we preadministered non-PEGylated liposomes to BALB/c mice, and then studied the biodistribution of the test-dose PEGylated liposomes. As shown in Fig. 5, the hepatic uptake and the clearance from bloodstream of the test-dose PEGylated liposomes were increased dramatically in the mice pretreated with non-PEGylated liposomes, as in the case of pretreatment with PEGylated liposomes. This indicates that the ABC phenomenon was thus induced regardless of modification of the liposomes with PEG chains.

3.6. Time-dependency of anti-PEG IgM antibody production

We next determined the time-dependency of the production of anti-PEG IgM antibody in BALB/c mice. The amount of anti-PEG IgM antibody was determined at days 1, 3, 7, 11, 14, 21 and 27 after pretreatment with PEGylated liposomes, PEG–Dox liposomes, or PBS. Anti-PEG IgM antibody production reached maximum level about 3 days after the administration of PEGylated liposomes (Fig. 6). This transient increase in IgM antibody like T cell-independent B cell response was observed in this experiment. On the contrary, the PBS and PEG–Dox liposomes groups showed little production of anti-PEG IgM antibody.

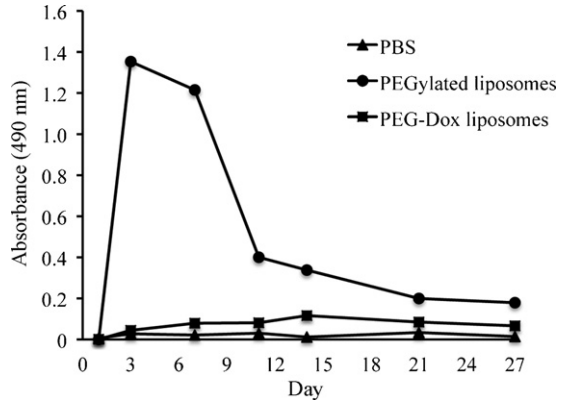

Fig. 6. Time-course of anti-PEG IgM antibody production. BALB/c mice were intravenously injected with PBS, PEGylated liposomes or PEG–Dox liposomes. At days 1, 3, 7, 11, 14, 21, and 27 after the injection, serum samples were collected. Anti-PEG IgM antibody was then detected by ELISA.

4. Discussion

The ABC phenomenon implies caution about the pharmacokinetics of nanoparticles in the case of repeated injection of nanomedicines, especially in the clinical setting. Thus, elucidating the mechanism of the ABC phenomenon is important for the development of DDS (drug delivery system) drugs. In this study, we determined the biodistribution of test-dose PEGylated liposomes in mice preadministered PEG–Dox liposomes or a mixture of free Dox and "empty" PEGylated liposomes. We and Laverman et al. previously reported that the ABC phenomenon was not induced when the rats were pretreated with PEG–Dox liposomes (Ishida et al., 2006a,b,c; Laverman et al., 2001). These reports indicated that preadministration of PEG–Dox liposomes could abolish the induction of the ABC phenomenon, although the effect of the mixture of free Dox and "empty" PEGylated liposomes has not been clarified. The production of anti-PEG IgM antibody was increased by the administration of the mixture of free Dox and "empty" PEGylated liposomes in a manner similar to that of PEGylated liposomes, but no increase was seen with PEG–Dox liposomes. These data suggest the importance of Dox delivery to immune cells by PEGylated liposomes, and indicate that Dox encapsulated in the liposomes was delivered to the immune cells responsible for the ABC phenomenon. And then, Dox that had been taken up into the immune cells could induce apoptosis of them, resulting in no induction of the IgM antibody against PEGylated liposomes and ABC phenomenon. In general, free Dox causes adverse reactions such as leucopenia and cardiotoxicity. Liposomal Dox was developed to reduce these side effects, which could significantly increase the tolerable dosage (O'brien et al., 2004). If the induction of the ABC phenomenon was canceled by a non-specific effect of Dox, the mixture of free Dox and "empty" PEGylated liposomes would have abolished the induction of the phenomenon. Accordingly, we conclude that the encapsulation of cytotoxic agents in PEGylated liposomes prevents the induction of the ABC phenomenon.

The ABC phenomenon was observed in the BALB/c *nu*/*nu* mice, but not in the BALB/c SCID mice. In addition, production of anti-PEG IgM antibody was increased by the injection of PEGylated liposomes in BALB/c *nu*/*nu* mice but not in BALB/c SCID mice at all. These results suggest that the induction of ABC phenomenon is independent of the T cells, but dependent on B cells. On the other hand, the phenomenon was also induced by the preadministration of non-PEGylated liposomes, suggesting that the specificity of the IgM antibody would be comparatively low. In this point, we previously reported that the administration of PEGylated liposomes triggered IgM antibody production against both PEG moieties and lipid moieties in rats, although the reactivity of IgM antibody against the latter was not so high (Wang et al., 2007). Anti-PEG IgM antibody produced by preadministration of PEGylated liposomes was transiently increased and then decreased rapidly. These data suggest that PEGylated liposomes might be recognized as a TI-2 antigen.

In general, most antigens in the natural world are T cell-dependent antigen. In the immune response to them, naïve B cells can differentiate into immunoglobulin-secreting cells or they can seed a germinal center and develop into memory B cells after exposure to a T cell-dependent antigen (Ahmed and Gray, 1996; Liu et al., 1988, 1991). However, there are other type antigens which are composed of repetitive structures such as polysaccharides or lipopolysaccharide derived from bacteria and belong to the TI-2 antigen category. These antigens activate B cells, which then produce antibodies such as IgM and IgG without the interaction with

helper T cells; and these IgM antibodies tend to have low affinity (Beringue et al., 2000). TI-2 responses are characterized by early B cell proliferation in all splenic compartments and differentiation into antibody-producing plasma cells starting at day 3 after immunization, but the number of plasma cells falls markedly during the second week after immunization (Garcia De Vinuesa et al., 1999). These TI-2 antigens induce only limited isotype switching and do not induce memory B cells (Beringue et al., 2000). In addition, B cells respond against TI-2 antigens and profoundly participate in the response of complements (Zandvoort and Timens, 2002). It is therefore considered that when PEGylated liposomes enter into the bloodstream, complement components may bind to the liposomal surface and activate B cells to secrete IgM antibodies such as anti-PEG IgM antibody. Marginal zone B (MZ-B) cells in the spleen and B-1B cells in the intraperitoneal cavity play a central role in the immune response against TI-2 antigen (Martin and Kearney, 2002). Since we have already reported that the phenomenon was not induced in rats that had had their spleens removed (Ishida et al., 2006a,b,c), we hypothesize that MZ-B cells recognize PEGylated liposomes as a TI-2 antigen and secrete anti-PEG IgM antibody. It appears that anti-PEG IgM antibody binds to the test-dose PEGylated liposomes and makes complexes with complement components. Then, these complexes accumulate in the liver, resulting in the rapid clearance of PEGylated liposomes.

Our present findings strongly suggest that PEGylated liposomes and conventional liposomes without PEG modification are recognized as TI-2 antigens and that the induction of the ABC phenomenon is related to a T cell-independent B cell response.

5. Conclusion

This study revealed that the ABC phenomenon occurred in BALB/c *nu*/*nu* mice, but not in BALB/c SCID mice. Anti-IgM antibody production, possibly derived from a T cell-independent B cell response, would be essential for the induction of the phenomenon. We anticipate that the elucidation of the ABC phenomenon will be helpful for the development of future DDS formulations.

Acknowledgments

This research was supported by global COE program. We thank Philip Hawke at the University of Shizuoka for helpful advice in writing the manuscript.

References

Ahmed, R., Gray, D., 1996. Immunological memory and protective immunity: understanding their relation. Science 272, 54–60.

Allen, T.M., 1994. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol. Sci. 15, 215–220.

Beringue, V., Demoy, M., Lasmezas, C.I., Gouritin, B., Weingarten, C., Deslys, J.P., Andreux, J.P., Couvreur, P., Dormont, D., 2000. Role of spleen macrophages in the clearance of scrapie agent early in pathogenesis. J. Pathol. 190, 495–502.

Berry, G., Billingham, M., Alderman, E., Richardson, P., Torti, F., Lum, B., Patek, A., Martin, F.J., 1998. The use of cardiac biopsy to demonstrate reduced cardiotoxicity in AIDS Kaposi's sarcoma patients treated with pegylated liposomal doxorubicin. Ann. Oncol. 9, 711–716.

Dams, E.T., Laverman, P., Oyen, W.J., Storm, G., Scherphof, G.L., Van Der Meer, J.W., Corstens, F.H., Boerman, O.C., 2000. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J. Pharmacol. Exp. Ther. 292, 1071–1079.

Garcia De Vinuesa, C., O'leary, P., Sze, D.M., Toellner, K.M., Maclennan, I.C., 1999. T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci. Eur. J. Immunol. 29, 1314–1323.

Ishida, T., Atobe, K., Wang, X., Kiwada, H., 2006a. Accelerated blood clearance of PEGylated liposomes upon repeated injections: effect of doxorubicin-encapsulation and high-dose first injection. J. Control. Release 115, 251–258.

Ishida, T., Harada, M., Wang, X.Y., Ichihara, M., Irimura, K., Kiwada, H., 2005. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. J. Control. Release 105, 305–317.

Ishida, T., Ichihara, M., Wang, X., Kiwada, H., 2006b. Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes. J. Control. Release 115, 243–250.

Ishida, T., Ichihara, M., Wang, X., Yamamoto, K., Kimura, J., Majima, E., Kiwada, H., 2006c. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J. Control. Release 112, 15–25.

Ishida, T., Maeda, R., Ichihara, M., Irimura, K., Kiwada, H., 2003a. Accelerated clearance of PEGylated liposomes in rats after repeated injections. J. Control. Release 88, 35–42.

Ishida, T., Masuda, K., Ichikawa, T., Ichihara, M., Irimura, K., Kiwada, H., 2003b. Accelerated clearance of a second injection of PEGylated liposomes in mice. Int. J. Pharm. 255, 167–174.

Ishida, T., Wang, X., Shimizu, T., Nawata, K., Kiwada, H., 2007. PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. J. Control. Release 122, 349–355.

Koide, H., Asai, T., Hatanaka, K., Urakami, T., Ishii, T., Kenjo, E., Nishihara, M., Yokoyama, M., Ishida, T., Kiwada, H., Oku, N., 2008. Particle size-dependent triggering of accelerated blood clearance phenomenon. Int. J. Pharm. 362, 197–200.

Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K., Papahadjopoulos, D., 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta 1070, 187–192.

Laverman, P., Carstens, M.G., Boerman, O.C., Dams, E.T., Oyen, W.J., Van Rooijen, N., Corstens, F.H., Storm, G., 2001. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J. Pharmacol. Exp. Ther. 298, 607–612.

Liu, Y.J., Oldfield, S., Maclennan, I.C., 1988. Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zones. Eur. J. Immunol. 18, 355–362.

Liu, Y.J., Zhang, J., Lane, P.J., Chan, E.Y., Maclennan, I.C., 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur. J. Immunol. 21, 2951–2962.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284.

Maeda, N., Takeuchi, Y., Takada, M., Sadzuka, Y., Namba, Y., Oku, N., 2004. Anti-neovascular therapy by use of tumor neovasculature-targeted long-circulating liposome. J. Control. Release 100, 41–52.

Martin, F., Kearney, J.F., 2002. Marginal-zone B cells. Nat. Rev. Immunol. 2, 323–335.

Muggia, F.M., 1999. Doxorubicin-polymer conjugates: further demonstration of the concept of enhanced permeability and retention. Clin. Cancer Res. 5, 7–8.

O'brien, M., Wigler, N., Inbar, M., Rosso, R., Grischke, E., Santoro, A., Catane, R., Kieback, D., Tomczak, P., Ackland, S., Orlandi, F., Mellars, L., Alland, L., Tendler, C., Group Caelyx Breast Cancer Study, 2004. Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX/Doxil) versus conventional doxorubicin for first-line treatment of metastatic breast cancer. Ann. Oncol. 15, 440–449.

Oku, N., Doi, K., Namba, Y., Okada, S., 1994. Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice. Int. J. Cancer 58, 415–419.

Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov Jr., A.A., Trubetskoy, V.S., Herron, J.N., Gentry, C.A., 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim. Biophys. Acta 1195, 11–20.

Urakami, T., Akai, S., Katayama, Y., Harada, N., Tsukada, H., Oku, N., 2007. Novel amphiphilic probes for [18F]-radiolabeling preformed liposomes and determination of liposomal trafficking by positron emission tomography. J. Med. Chem. 50, 6454–6457.

Van Rooijen, N., Van Nieuwmegen, R., 1980. Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. Immunol. Commun. 9, 243–256.

Wang, X., Ishida, T., Kiwada, H., 2007. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. J. Control. Release 119, 236–244.

Zandvoort, A., Timens, W., 2002. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. Clin. Exp. Immunol. 130, 4–11.