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Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes

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Abstract

Earlier we reported that PEGylated liposomes lose their long-circulating characteristic when they are administrated twice in the same animal with certain intervals (referred to as the accelerated blood clearance (ABC) phenomenon). We proposed that anti-PEG IgM, induced by the PEGylated liposomes, is responsible for the phenomenon, based on the observation that IgM thus produced selectively binds to the surface of PEGylated liposomes, subsequently leading to substantial complement activation. Interestingly, we found that under certain circumstances administration of conventional liposomes without PEG-coating also caused a strong ABC response upon injection of a second dose of PEGylated liposomes, but not of conventional liposomes. This suggests that also conventional liposomes not modified with PEG can promote an IgM response against PEG. We report here that, irrespective of the presence or absence PEG-coating, a single first dose of liposomes is capable of inducing a strong anti-PEG IgM response and, under certain circumstances, also weak responses against other lipid components. A good correspondence was observed between the amount of IgM associating with both PEGylated and conventional liposomes, concomitant complement activation triggered by those liposomes and the magnitude of the ABC phenomenon against those liposomes. Taken together, our results demonstrate that the ABC phenomenon is fully attributable to production of anti-PEG IgM by the first dose of liposomes and the subsequent complement activation upon a second dose of PEGylated but not conventional liposomes. Although the responsible immunogenic epitopes of the liposomes remain to be determined, the immunogenicity of 'empty' liposomes presents a serious concern in the development of liposomal formulations and their use in the clinic. Furthermore, our findings as described here raise important concerns with regard to the safety and efficiency of liposomes currently under development for clinical use.

Keywords: IgM; Polyethylene glycol (PEG); Accelerated blood clearance (ABC) phenomenon; Liposomes; Repeated injection; Complement system

1. Introduction

Liposomes which are sterically stabilized with surface-coupled polyethylene glycol (PEG) can enhance their lifetime and that of entrapped therapeutic agents in the blood circulation [1–3]. It is hypothesized that the presence of PEG on the liposome attracts a water shell to the liposomal surface, providing a steric barrier against opsonins and/or recognition by cells of mononuclear phagocyte system (MPS) [4–6]. This, in turn, results in a decrease in the elimination rate of liposomes from the blood stream.

We and others have found that an intravenous injection of PEGylated liposomes causes a second dose of PEGylated liposomes, injected a few days later, to lose their long-circulating

Abbreviations: AUC, the area under the blood concentration–time curve; ABC, accelerated blood clearance; CHOL, cholesterol; ^{3}H-CHE, ^{3}H-cholesterylhexadecyl ether; DGVB^{2+}, VBS containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, 5%; D-glucose and 0.1%; gelatin; DHPE, dihexadecanoylglycerophosphoethanolamine; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; mPEG$_{2000}$-DSPE,1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; MLV, multilamellar vesicles; MPS, mononuclear phagocyte system; PEG, polyethylene glycol; PL, phospholipid; SRBC, sheep red blood cells sensitized with monoclonal anti-sheep IgM antibody; TI antigen, a T-cell independent antigen or thymus-independent antigen; TI-1, type-1 TI antigen; TI-2, type-2 TI antigen; VBS, veronal-buffered saline.

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characteristics and accumulate extensively in liver, despite the presence of PEG on the surface of the liposomes [7–10]. This phenomenon is referred to as the "accelerated blood clearance (ABC) phenomenon". Although the details of underlying mechanism are not yet elucidated, we proposed the following tentative mechanism for the induction of the ABC phenomenon on the basis of our earlier results [11–13]: anti-PEG IgM, produced in the spleen in response to an injected dose of PEGylated liposomes, selectively binds to the PEG on a second dose of these liposomes, injected several days later, and subsequently activates the complement system. This, in turn, leads to opsonization of the liposomes by C3 fragments and, as a consequence, to enhanced uptake of the liposomes by the Kupffer cells in liver.

Earlier reports from our laboratory [11–14] indicate that 'empty' PEGylated liposomes are immunogenic and promote antibody, especially IgM, responses against a second dose of such liposomes. This suggests that any PEGylated liposomal formulation, even if containing non-immunostimulatory payloads such as cytotoxic agents [14], may display unexpected pharmacokinetic behavior upon repeated injection and, as a consequence, may show less therapeutic efficacy or even cause undesirable side effects. Therefore, a strategy to abrogate the immunogenicity of PEGylated liposomes without significantly compromising their in vivo performance would be highly desirable for the further development of this otherwise promising drug delivery system. Therefore, studies providing further insight in the mechanisms underlying the ABC phenomenon are of great importance.

We have shown that anti-PEG IgM is responsible for the induction of the ABC phenomenon in rats [12,13]. Similar results had been reported. Judge et al. [15] showed that anti-PEG antibodies were induced after injection of plasmid DNA-containing PEGylated liposome in mice. Srode et al. [16] showed that anti-PEG IgG was induced in response to the first injection of empty PEGylated liposome in rabbits. Both reports demonstrated that the induction of anti-PEG antibodies causes accelerated blood clearance of subsequently injected PEGylated liposomes. However, a clear-cut relationship between the amount of anti-PEG antibodies associated with PEGylated liposomes and the degree of induced ABC phenomenon has not been established. In addition, our earlier studies revealed that, at higher lipid dose (5 μmol phospholipid (PL)/kg), even conventional liposomes (without PEG-coating) can induce enhanced clearance of a subsequently injected dose of PEGylated, but not conventional, liposomes [17]. This raised the question if anti-PEG IgM is also produced following injection of conventional liposomes, causing accelerated clearance of a subsequent dose of PEGylated liposomes. In the present study we therefore addressed these issues. Our findings raise important concerns regarding the safety and efficiency of PEGylated liposomes currently in use or under consideration for clinical application.

2. Materials and methods

2.1. Materials and animals

Hydrogenated egg phosphatidylcholine (HEPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) and DSPE were generously donated by Nippon Oil and Fat (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. Sepharose 4 Fast Flow was purchased from Amersham-Pharmacia Biotech (Upsala, Sweden). Rhodamine-derivatized dihexadecanoylglycerophosphoethanolamine (DHPE) was purchased from Molecular Probes (OR, USA). ^{3}H-Cholesterylhexadecyl ether (^{3}H-CHE) was purchased from NEN Research Products (MA, USA). All other reagents were of analytical grade.

Male Wistar rats weighing 250–300 g were purchased from Japan SLC (Shizuoka, Japan). Upon arrival, the rats were allowed to acclimatize for at least 1 week. They had free access to water and rat chow, and were housed under controlled environmental conditions (constant temperature, humidity, and a 12-h dark–light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of The University of Tokushima.

2.2. Preparation of liposomes

PEGylated liposomes were composed of HEPC:CHOL:$mPEG_{2000}$-DSPE (1.85:1.0:0.15 molar ratio). Conventional liposomes were composed of HEPC:CHOL (2:1 molar ratio). Liposomes were prepared as previously described [10]. Briefly, the lipids were dissolved in chloroform and, after evaporation of the organic solvent, the resulting lipid film was hydrated in HEPES buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4) to produce multilamellar vesicles (MLV). The MLV were sized by repeated extrusion through polycarbonate membrane filters (Nuclepore, CA., USA) with consecutive pore sizes of 400 (×3), 200 (×3), 100 (×5) and 80 (×3) nm. The mean diameters of the prepared liposomes were determined by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The mean diameters for the PEGylated liposomes and conventional liposomes were 115±11 nm (n=5) and 109±13 nm (n=5), respectively. The concentration of phospholipid was determined by colorimetric assay [18]. To follow the biodistribution of the second dose of liposomes, the liposomes were labeled with a trace amount of ^{3}H-CHE (40 μCi/μmol lipid) as a nonexchangeable lipid phase marker. To collect liposome fractions following gel chromatography, the liposomes were labeled with a trace amount of rhodamine-labeled DHPE.

2.3. Biodistribution and pharmacokinetics of second (test) dose liposomes

For the first injection, the liposomes (PEGylated or conventional), at a dose of 5 or 0.001 μmol PL/kg, was injected via the femoral vein under ether anesthesia and sterile treatment. Control animals received HEPES buffered saline instead of liposomes. In all experiments, to determine the biodistribution of subsequently injected test liposomes (PEGylated or conventional), pre-treated rats were cannulated via the left femoral vein (PE-20, Natsume, Tokyo, Japan) and artery (PE-50) under ether anesthesia and sterile treatment. Then, ^{3}H-CHE-labeled test

liposomes were injected intravenously at a dose of 5 μmol PL/kg via a cannula positioned in the femoral vein. At selected post-injection time points (2 min, 30 min, 1 h, 2 h, 4 h, 10 h and 24 h), blood (100 μl) was sampled through a cannula in the femoral artery. The liver and spleen were removed, after withdrawing the last blood sample at 24 h. Radioactivities in blood and tissues were assayed as described previously [19]. Pharmacokinetic parameters were calculated using polyexponential curve fitting and the least-squares parameter estimation program PKAnalyst (Micromath, UT, USA). The hepatic clearance was calculated as follows:

$$\mathrm{CLh} = X_{(24)}/\mathrm{AUC}_{(0\rightarrow 24)}$$

where $X_{(24)}$ is the amount of test liposomes accumulated in liver at 24 h post-injection. $\mathrm{AUC}_{(0\rightarrow 24)}$ is the area under the blood concentration–time curve from time 0 to 24 h post-injection.

2.4. Determination of serum proteins associated with lipoosmes

2.4.1. In vitro liposome/serum incubation

To 300 μl of liposome suspension (15 mM), 1200 μl of 100% rat serum was added in 1.5 ml polypropylene micro test tubes (Eppendorf, Hamburg, Germany) and the liposome/serum mixture was incubated for 15 min at 37 °C at 80% final serum concentration. The incubation mixture was immediately cooled for 5 min using an ice/water bath to stop the reaction. Rat sera were prepared from rats that had been treated with either PEGylated liposomes (0.001 or 5 μmol PL/kg), conventional liposomes (0.001 or 5 μmol PL/kg) or HEPES buffered saline 5 days earlier. Collected sera were stored at −80 °C until use.

2.4.2. Isolation of liposomes from the incubation mixture

To separate liposomes from serum, including very low density and low density lipoproteins, the incubation mixture (1500 μl) prepared above was loaded onto a 1.5 cm × 30 cm Sepharose 4 Fast Flow column and eluted with HEPES buffered saline at a flow rate of 1.25 ml/min. To determine the liposome concentration in the fractions, each fraction (600 μl) was analyzed for fluorescence intensity of rhodamine-DHPE in the liposomes at excitation and emission wavelengths of 530 and 590 nm respectively, using a Wallac1420 ARVOsx (PerkinElmer Life Science, MA, USA). The fractions with the highest fluorescence intensity, typically fractions 6 and 7 from each column, were pooled and concentrated using an Amicon Ultra-15 Centrifugal Filter Device (Millipore, MA, USA). The phospholipid concentration in the concentrated liposomes was determined as described above. The samples were stored at −80 °C until use.

2.4.3. Quantitative determination of IgM and IgG

175 μl of sample (4 μmol total lipid/ml) was mixed with 25 μl of 20% Triton X-100 to dissolve the liposomes. The resulting solution was used for ELISA after a 1:100 dilution with sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0). The Triton X-100 in the sample did not interfere with the ELISA system under our experimental conditions.

Quantification of IgM and IgG was performed using the Rat IgM ELISA Quantitation Kit and the Rat IgG ELISA Quantitation Kit of BETHYL Laboratories, TX, USA. Briefly, 100 μl of capture antibody (10 μg/ml, Goat anti-Rat IgM IgG or Goat anti-Rat IgG IgG) in coating buffer (50 mM Carbonate–Bicarbonate, pH 9.6) was added to each well of a 96 well-plate (EIA/RIA plate, Corning, NY, USA) and incubated for 1 h. After incubation, the wells were washed three times with the wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Then the blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was added to each well and the plate was incubated for 30 min. After incubation, the wells were washed three times with the wash solution and 100 μl of standard or sample in the sample diluent was added to the wells. After incubation for 60 min, the wells were washed five times with the wash solution and 100 μl of hoseradish peroxidase (HRP)-conjugated antibody (1 μg/ml, Goat anti-Rat IgM IgG-HRP conjugate or Goat anti-Rat IgG IgG-HRP conjugate) in the conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) was added to each well. After incubation for 1 h, the wells were washed 5 times with the wash solution. The coloration was initiated by adding *o*-phenylene diamine (1 mg/ml) (Sigma, MO, USA). After 15 min incubation, the reaction was stopped by adding 100 μl of 2N H_2SO_4 and the absorbance was measured at 490 nm using a Microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were carried out at room temperature.

2.5. Anti-lipid IgM ELISA

Rats were intravenously injected with PEGylated liposomes (0.001 μmol or 5 μmol PL/kg), conventional liposomes (0.001 or 5 μmol PL/kg) or HEPES buffered saline. 5 days later, rats were sacrificed and blood was withdrawn. Serum was collected after centrifugation (15 min, 4 °C, 3000 rpm).

An ELISA was employed to detect IgM against the PEG-lipid and other lipid components of PEGylated liposomes or conventional liposomes using methods described earlier with minor modification [15,20]. All incubations were carried out at room temperature. 10 nmol of lipid (HEPC, CHOL, DSPE or mPEG_{2000}-DSPE) in 50 μl of 100% ethanol was added to 96-well plates (EIA/RIA plate, Corning, NY, USA). The plates were allowed to air dry completely for 2 h. Then, diluted rat serum was incubated in the prepared plate for 1 h. Following processes were same as the processes described in 2.4.3.

2.6. Measurement of complement activity

As a measurement of residual serum complement activity, the functional complement assay (CH-50) was performed according to the modified method of Kabat and Mayer [21] with sheep red blood cell sensitized with monoclonal anti-sheep IgM antibody (SRBC) (CH50 SEIKEN, DENKA SEIKEN, Tokyo, Japan).

Briefly, 90 μl of various rat sera was incubated with 10 μl of liposomes (2.5 mM) at 37 °C for 15 min. As a control, 90 μl of naïve serum was incubated with 10 μl of HEPES buffered saline

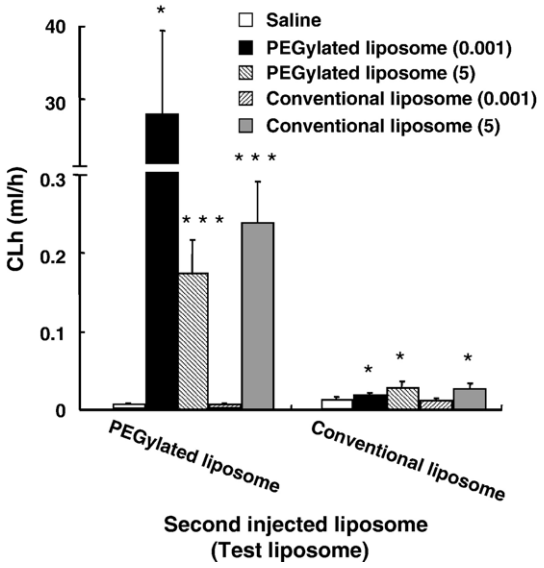


Fig. 1. Hepatic clearance (CLh) of second-dose PEGylated or conventional liposomes. Radio-labeled PEGylated or conventional test-dose liposomes were intravenously injected on day 5 post-first injection. Type and dose of the first-dose liposomes are presented in the Figure. Rats receiving saline as a first injection served as controls. Each value represents the mean±S.D. of three separate experiments. *P* values apply to differences control group (saline). **P*<0.05, ****P*<0.005.

instead of liposomes. Then, 10-µl aliquots of treated serum were diluted with 1 ml of veronal-buffered saline (VBS) composed of 150 mM NaCl and 75 mM sodium barbital (pH 7.4) containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, 5% D-glucose and 0.1% gelatin ($DGVB^{2+}$). 400 µl of SRBC (5×10^8/ml) was mixed with 2.6 ml of sequentially diluted treated serum and incubated at 37 °C for 1 h. Unlysed SRBC were pelleted by centrifugation (3000 rpm, 30 min, 4 °C) and the amount of hemoglobin released into the supernatant was quantified spectrophotometrically at 541 nm (UV-1600, Shimadzu, Kyoto, Japan). The CH-50 was calculated according to the equations recommended by the manufacturer.

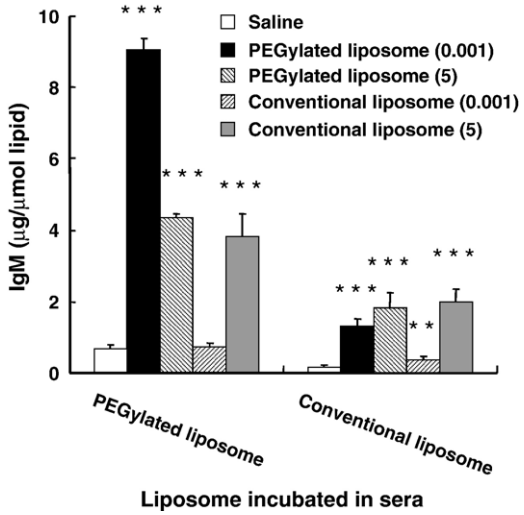


Fig. 2. Association of IgM with PEGylated or conventional liposomes following incubation in various sera. PEGylated or conventional liposomes were injected intravenously in rats and 5 days later sera were collected. Liposome type and dose (µmol PL/kg) are specified in the Figure. Liposomes, either PEGylated or conventional, were incubated in the different sera as detailed in the Methods section. After incubation for 15 min, the incubation mixture was chromatographed on a Sepharose 4 Fast Flow column. IgM in the fractions containing liposomes was quantitatively determined. Each value represents the mean±S.D. of three separate experiments. *P* values apply to differences against the control group (saline). ***P*<0.01, ****P*<0.005.

2.7. Statistics

All values are expressed as the mean±S.D. Statistical analysis was performed with a two-tailed unpaired *t* test using GraphPad InStat software (GraphPad Software, CA, USA). The level of significance was set at $P<0.05$.

3. Results

3.1. Hepatic clearance of a second dose of PEGylated or conventional liposomes

After induction of the ABC phenomenon, a subsequent dose of PEGylated liposomes is rapidly cleared by Kupffer cells in the liver [7,11]. Hepatic clearance reflects the liposome uptake activity of the Kupffer cells and hence is a good parameter of the magnitude of the induced ABC phenomenon [9,10]. As shown in Fig. 1 (open bars, pre-injection with saline), hepatic clearance of a single dose of test PEGylated or conventional liposomes is very low. Upon a pre-injection with low- or high-dose PEGylated liposomes or with high-dose conventional liposomes, hepatic clearance of a subsequent dose of PEGylated liposomes 5 days later increased dramatically, for the low-dose PEGylated liposomes culminating in a several orders of magnitude increase from 0.0045 to 28.5 ml/h (Fig. 1, open vs. solid bar on the left). More moderate but still considerable

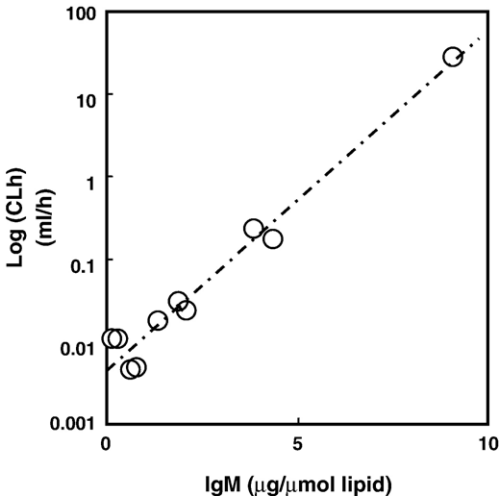
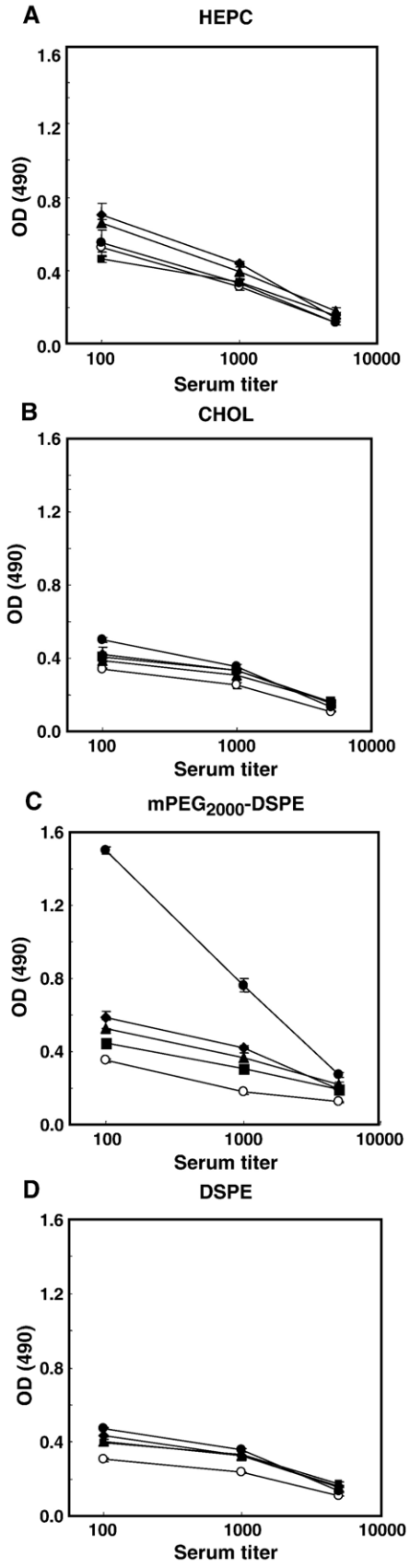


Fig. 3. Correlation between CLh of test liposomes and amounts of liposome-bound IgM. The 10 values found for the CLh in Fig. 1 were plotted against the corresponding 10 values found for the amount of liposome-bound IgM in Fig. 2. "Corresponding values" are values found for the two plotted parameters (i.e. hepatic clearance and amount of IgM) under matching experimental conditions.



increases in hepatic clearance of PEGylated liposomes were observed upon pre-injection with high-dose PEGylated liposomes (hatched bar on the left, 0.17 ml/h) or conventional liposomes (grey bar on the left, 0.24 ml/h). When using conventional liposome as the second test dose (all bars on the right-hand side), very low hepatic clearance values were observed, similar to the value found for a single injection (0.011 ml/h), irrespective of lipid dose or liposome type used for the first injection.

3.2. In vitro quantitative determination of IgM associating with liposomes

The left part of Fig. 2 shows that, when PEGylated liposomes were incubated with sera from rats pre-dosed with PEGylated liposomes, IgM binding was several-fold increased as compared to incubation with naïve serum (rats pre-dosed with saline). Remarkably, the level of binding was inversely related to the size of the pre-dose: the low pre-dose (0.001 μmol PL/kg) producing almost 9 μg of IgM per μmol of PL and the high pre-dose (5 μmol PL/kg) only about 4 μg/μmol PL. Predosing with conventional liposomes only led to substantial IgM binding when the high dose (5 μmol PL/kg) was administered. When conventional liposomes were incubated in different sera (Fig. 2 right part), moderate IgM binding was observed in sera from rats pre-dosed with PEGylated liposomes virtually irrespective of the size of the pre-dose. Of the sera from rats that were pre-dosed with conventional liposomes only the serum from the high pre-dose rats (5 μmol PL/kg) showed significant IgM binding. Apparently, the steric barrier provided by the PEG layer does not prevent abundant binding of the IgM to the liposomal surface when the liposomes are exposed to sera from rats pre-dosed with PEGylated or conventional liposomes. Compared to this, IgM binding to conventional liposomes was much lower in all sera.

On the other hand, throughout the experiments, with all sera negligible IgG binding was observed on both PEGylated and conventional liposomes (not shown). This observation is consistent with our earlier results with Western blotting [13] and quantitative ELISA [12].

3.3. Correlation between IgM binding and hepatic clearance

In Fig. 3, the hepatic clearance values as presented in Fig. 1 were plotted against the amounts of liposome-associated IgM presented in Fig. 2. The two parameters correlated quite well (correlation efficiency (r^2)=0.966), indicating that the IgM associating with the liposomes plays a crucial role in the ABC phenomenon.

Fig. 4. IgM titers of sera against individual lipid components of PEGylated or conventional liposomes. Titers of IgM reactive against individual lipid components of both types of liposomes were determined in the sera collected at 5 day after the first injection. Sera were pooled from rats treated with HEPES buffered saline (○), PEGylated liposomes (0.001 μmol/kg) (●), PEGylated liposomes (5 μmol/kg) (▲), conventional liposomes (0.001 μmol/kg) (■) or conventional liposomes (5 μmol/kg) (⧫).

3.4. Specificity of liposome-reactive IgM

To evaluate the specificity of the liposome-reactive IgM shown in Fig. 2, a modified ELISA was employed. The specificity of the IgM in the collected sera was examined by incubating the sera in wells coated with a lipid component of liposome; HEPC (Fig. 4A), CHOL (Fig. 4B), $mPEG_{2000}$-DSPE (Fig. 4C) or DSPE (Fig. 4D), the lipid anchor of $mPEG_{2000}$-DSPE. In the sera from rats treated with HEPES buffered saline, low IgM binding to all individual lipid components was observed (open circles). In the sera from rats pre-treated with low-dose PEGylated liposomes, substantial excess IgM binding, as compared to the sera from saline-pretreated animals, was observed only in the $mPEG_{2000}$-DSPE-coated wells. Very small increments of IgM binding were detected in the CHOL and DSPE-coated wells while virtually no excess binding to the HEPC- coated wells was observed. In the sera from rats pre-treated with high-dose PEGylated liposomes, small but significant increments in IgM binding were observed in the HEPC, $mPEG_{2000}$-DSPE and DSPE- coated wells, while no excess binding was found in the CHOL-coated wells. In the sera from animals pre-treated with low-dose conventional liposomes small but significant increases in $mPEG_{2000}$-DSPE and DSPE-reactive IgM were detected, while no increase in HEPC and CHOL-reactive IgM was found. In the sera from rats pre-treated with high-dose conventional liposomes small amounts of HEPC, $mPEG_{2000}$-DSPE and DSPE-reactive IgM but no CHOL-reactive IgM's were detected.

3.5. Complement activation mediated by IgM associated with the liposomes

It is well known that IgM is one of the major opsonins capable of facilitating clearance of foreign materials such as invading pathogens. By itself, however, IgM has no ability to promote endocytosis or phagocytosis of foreign materials directly. It indirectly enhances the clearance of foreign materials as a consequent of complement activation. Therefore, we recently proposed that liposome-reactive IgM enhances the rapid uptake of subsequently injected PEGylated liposomes via complement activation *in vivo* [13]. We therefore investigated if complement activation is induced by liposome-reactive IgM.

Following incubation with PEGylated liposomes, significant complement activation was observed in sera from rats pre-treated with PEGylated liposomes at lower and higher lipid doses (0.001 and 5 μmol PL/kg) (Fig. 5A). This is consistent with our earlier observation [13]. In sera from rats pre-treated with conventional liposomes, complement activation by PEGylated liposome was observed only in the sera from animals pre-treated with the higher lipid dose (5 μmol PL/kg) (Fig. 5A). The levels to which PEGylated liposomes produced complement activation in sera from rats pre-treated with the two different types and doses of liposomes decreased in the following order: low-dose PEGylated > high-dose conventional > high-dose PEGylated > low-dose conventional (Fig. 5A). Following incubation with conventional liposomes, only a low level of complement activation was observed in the three sera

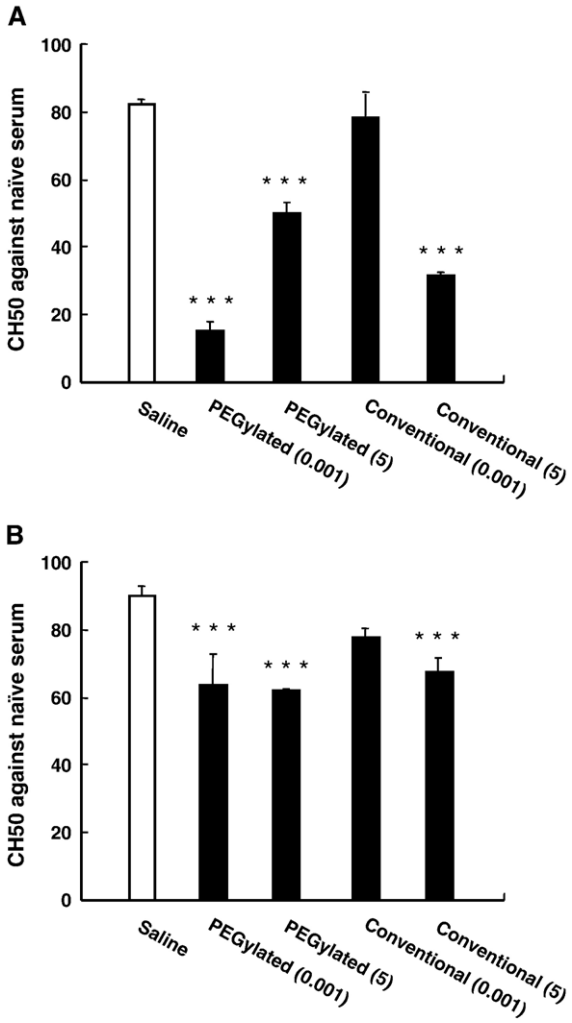


Fig. 5. Complement activation induced by PEGylated liposome or conventional liposome in serum. Sera were obtained from rats injected with PEGylated or conventional liposomes 5 days earlier. Following incubation with PEGylated liposomes (A) or conventional liposomes (B) residual complement activity in the various sera was assayed as described in the Materials and methods section. Type and lipid dose of the injected liposomes are specified in the Figure. The values represent the mean ± S.D. of three independent experiments. *P* values apply to differences against control group (saline). ***$P<0.005$.

from rats pre-dosed with low and high-dose PEGylated liposomes and with high-dose conventional liposome (Fig. 5B). We found no significant difference between those sera. Pre-treatment of rats with low-dose conventional liposomes failed to produce significant complement activation potential in the serum.

4. Discussion

The results described here clearly indicate that pre-administration of liposomes promotes the production of anti-liposome IgM regardless of the presence or absence of PEG-coating on their surface (Fig. 2). We demonstrated that the major liposomal component responsible for the recognition of the PEGylated liposomes by the anti-liposome IgM is the PEG moiety, in line with the observation that also the enhanced clearance phenomenon

observed with test-dose liposomes is specifically seen with PEGylated but not with conventional liposomes. We propose that the enhanced clearance of the PEGylated liposomes is caused by IgM-induced complement activation and subsequent complement-mediated elimination. An increase in the promotion of anti-PEG IgM production could be the trigger that converts the PEGylated liposome from a non-harmful into a potentially harmful pathological formulation.

Cheng and co-workers [22,23] demonstrated that a monoclonal antibody (IgM) against PEG obtained following immunization with PEGylated β-glucuronide recognizes the repeating $O–CH_2–CH_2$ subunit (16 units) of PEG. Thus, the anti-PEG IgM described in this study, if it is monoclonal, may bind to the similar epitope. Generally, IgM antibodies tend to be of low affinity. However, IgM molecules form pentamers whose ten antigen-binding sites can bind simultaneously to multivalent antigens. This compensates for the relatively low affinity of the IgM monomers by multipoint binding, conferring high overall avidity. The high avidity, a basic characteristic of IgM, might provide sufficiently strong binding of the generated anti-PEG IgM to the PEGylated liposome, specifically to the PEG moiety, to overcome the protective effect and the free movement of the PEG chains on the liposome surface.

Although our results indicate that the majority of the generated IgM is a specific anti-PEG IgM antibody (Fig. 4), we can not exclude the possibility that in addition IgMs reactive against other lipid components or even against the liposome as such were elicited by the pre-administration of liposomes and that such IgMs are involved as well in the enhanced clearance of subsequently injected liposomes. The pre-administration of PEGylated liposomes caused the production of IgM reactive against not only PEGylated liposomes but also against conventional liposomes (Fig. 2). The IgM formed also contained some anti-HEPC IgM activity (Fig. 4). In addition, pre-administration of conventional liposomes produced IgM reactive against similar liposomes, probably against the HEPC (Figs. 2 and 4). This might imply that the antibody (IgM) responses against the injected liposomes are polyclonal. We consider it most likely that the anti-HEPC IgM generated mainly accounts for the accelerated clearance of conventional liposomes, but it may contribute to the enhanced clearance of PEGylated liposomes as well.

As described in Fig. 3, there was a tight correlation between hepatic clearance values and the amounts of bound IgM. In addition, the magnitude of complement activation by the liposomes correlated well with the amount of IgM associating with the liposomes (Figs. 2 and 5). These findings strongly suggest that it is the binding of the IgM to the second dose of injected liposomes and the subsequent activation of complement that leads to the enhanced clearance of the liposomes in the ABC phenomenon. This supports our previously proposed mechanism for the induction of the phenomenon [12,13]. The quantity of the anti-PEG IgM or anti-PC IgM produced may be a determinant in inducing the phenomenon. Earlier reports [7–11,17,24] demonstrated that various factors, such as dose and physicochemical properties of the initially injected liposome, the interval between injections and animal species remarkably influence the magnitude of induced the ABC phenomenon. This could be explained by assuming that all these factors may affect the IgM responses against the injected liposomes.

It is generally considered that ‘empty’ liposomes without encapsulated or surface-coupled proteins are immunologically inert. Our current and earlier results, clearly indicating that ‘empty’ liposomes are immunogenic and promote a strong IgM response [13], are contradictory to this. Recent studies from our laboratory indicated that the spleen plays an important role in promoting the formation of IgM reactive against PEGylated liposomes [12,14]. In addition, we showed that the immune reaction in the spleen against the PEGylated liposomes extends over a period of at least 2–3 days following the first administration and then IgM reactive against PEGylated liposomes is produced [12]. It has been reported that B cells in spleen are responsible for the first line of defense and able to produce large amounts of neutralizing antibodies in a short period (3–4 days) [25,26]. Accordingly, B-cells in the spleen may also play an important role in the induction of the ABC phenomenon.

A remarkable observation done in this study was that conventional liposomes, despite the lack of a PEG-coating, produced a significant level of anti-PEG IgM (Figs. 2 and 4). This suggests that the antigenic epitope capable of generating anti-PEG IgM is not only present in the PEG moiety but is also displayed in other components of the liposome or on the liposomal structure as such. The immune responses related to the ABC phenomenon are likely to be part of the innate immune system, because the induction of the phenomenon was completed within 5 days post-first injection of either conventional [11] or PEGylated liposomes [13]. B cells belonging to the innate immune system can directly interact with invasive pathogens and viruses through immunoglobulin (IgM) receptors with an infinite number of unique specificities and produce IgMs and IgGs which have broad specificities [27]. Thus, the IgM response observed in this study might be the result of non-specific stimulation to B cells by a structural component(s) of the conventional liposome or by the liposome itself. The mechanism by which conventional liposomes without PEG-coating can elicit an anti-PEG IgM response remains unclear in this stage.

Liposome must be classified as a T-cell independent (TI) antigen (or Thymus independent antigen) since there are no peptide components that can be presented by helper T cells. TI antigens can activate B cells and be induced to synthesize IgM antibodies at the early stage of immunization [27–29]. The antigens generally fall into two classes (TI-1 or TI-2) which activate B cells by two different mechanisms. TI-1 antigens, such as bacterial lipopolysaccharides, at higher concentration are potent B-cell (mature and immature) mitogens, capable of non-specific, polyclonal activation of B cells and, in turn, polyclonal IgM production and, at lower concentration, are capable of specifically activating B cells, eliciting a specific antibody response [27–29]. In contrast, TI-2 antigens only activate mature B cells and consist of highly repetitive structures, such as capsular polysaccharide [27–29]. In addition, these TI antigens induce only limited isotype switching and do not induce memory B cells that produce IgG

antibodies [27]. Recently, similar T cell-independent immune responses were reported in mice immunized with CpG ODN-containing liposomes [30], ODN-containing PEGylated liposomes [31] and pDNA-containing PEGylated liposomes [15]. Repeating polymers such as PVP (polyvinylpyrrolidone) and liposomes were shown as the examples of TI-2 antigens in the early study [32]. Although precise studies are required, it can be assumed that the PEGylated liposomes (liposome coated with repeating polymer-PEG) may function as a TI-2 antigen.

It is assumed that, after an intravenous administration of liposomes, serum opsonins such as C-reactive protein [33], fibronectin [33] and β2-glycoprotein I [34], are adsorbed onto the surface of the liposomes by a process called opsonization, triggering recognition and uptake of liposomes by the cells of the MPS. Remarkable increases in the serum levels of some of these opsonins are observed in certain conditions such as the acute phase response. Thus, other serum factor(s) than complement may also contribute to promote the ABC phenomenon. On the other hand, this is contradictory to earlier results reported by us showing by LC-MS/MS and Western blotting that IgM is the major serum protein associating with PEGylated liposomes displaying a strong induction of the ABC phenomenon [13]. Several studies have demonstrated the presence of naturally occurring anti-lipid antibodies (against phosphatidylcholine, phosphatidylethanolamine and CHOL [35,36]) in blood. However, in the present study, no significant increase in the level of antibodies against HEPC and CHOL was detected following pre-administration of liposomes (Fig. 4), suggesting that such antibodies are not a major trigger of the phenomenon.

5. Conclusion

The results presented here demonstrate that i.v. injected liposomes, regardless of the presence of a PEG-coating, are capable of inducing strong IgM responses against the PEG moiety of PEGylated liposomes and weak responses against the other lipid components. Although the exact mechanism of the induction the ABC phenomenon is not yet clear, our results clearly indicate that the production of such IgM is a major trigger transforming a subsequently injected dose of PEGylated liposomes from a less harmful into a harmful formulation. In turn, animals receiving a pre-dose of the liposomes acquire the ability to eliminate a second dose of PEGylated liposomes via complement activation. The immunogenicity of 'empty' liposomes is a serious concern in the development of liposomal formulations and their use in the clinic. A strategy needs to be developed to mitigate or even entirely abrogate the immunogenicity of the liposomes without significantly compromising their in vivo performance. The studies reported here may provide an important contribution to this task.

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