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PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner

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Abstract

We recently reported that intravenous injections of "empty" PEGylated liposomes without encapsulated or surface coupled proteins elicit a PEG-specific IgM response in rats. In the present study, simultaneous weak anti-PEG IgG and strong anti-PEG IgM responses were detected following intravenous injections of "empty" PEGylated liposomes. The pattern of immune response appears to differ from the classic primary response against T cell-dependent (TD) antigens. The anti-PEG IgM response was detected in T-cell deficient nude BALB/c mice following intravenous injection of "empty" PEGylated liposomes, suggesting that "empty" PEGylated liposomes initiate the immune response against PEG in a T cell-independent manner. In vitro splenic lymphocytes-proliferation assay indicated that TNP-LPS, a typical type 1 T cell-independent (TI) antigen (TI-1 antigen), significantly primed the proliferation, while TNP-Ficoll, a typical type 2 TI antigen (TI-2 antigen), and "empty" PEGylated liposomes did not prime any proliferation under these experimental conditions. In addition, in splenic marginal zone (MZ) B-cell-depleted rats, the anti-PEG IgM response was diminished, while the immune reactions against TNP-BSA (a TD antigen) and TNP-LPS (TI-1 antigen) were not diminished. These results demonstrate that "empty" PEGylated liposomes may promote the immune response against PEG as a result of priming the activation of MZ B cells, as TI-2 antigen promotes a specific IgM response. In conclusion, although the mechanistic details behind the immune reaction against "empty" PEGylated liposomes are not yet clear, the liposomes elicit an anti-PEG IgM response in a T cell-independent manner and appear to be a TI-2 antigen, and splenic MZ B cells may be essential for the immune response against "empty" PEGylated liposomes.

Keywords: PEGylated liposome; Polyethylene glycol (PEG); Anti-PEG IgM; Accelerated blood clearance (ABC) phenomenon; T cell-independent (TI) immune response

1. Introduction

Long-circulating PEGylated liposome has been widely used as a carrier in drug delivery to improve the lifetime of liposome entrapped therapeutic agents [1–3]. It is generally accepted that the presence of PEG (polyethylene glycol) on the surface of liposome attracts a water shell, providing a steric barrier against plasma proteins or opsonins [4–6]. This, in turn, results in a decrease in both the rate and extent of uptake of liposomes by the mononuclear phagocyte system. Despite this defined concept, it has been observed that unexpected alteration in the pharmacokinetic behavior of PEGylated liposomes occurred when it was injected repeatedly into mice, rats and rhesus monkeys, at certain intervals (referred to as the "accelerated blood clearance (ABC) phenomenon"). An intravenous injection of PEGylated liposomes cause a second dose of PEGylated liposomes, injected a few days later, to lose its long-circulating characteristics and accumulate extensively in the liver [7–10]. On the basis of our further studies [11–13], the following tentative mechanism for the induction of the ABC phenomenon was proposed: anti-PEG IgM, produced in the spleen in response to an injected first dose

Abbreviations: Ab, antibody; ABC, accelerated blood clearance; CHOL, cholesterol; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; LPS, Lipopolysaccharide; MZ, marginal zone; $mPEG_{2000}$-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy(polyethylene glycol)-2000]; PEG, polyethylene glycol; ODN, oligodeoxynucleotide; TD, T cell-dependent; TI, T cell-independent; TNP, 2,4,6-trinitrophenyl-aminoethylcarboxymethyl.

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of "empty" PEGylated liposomes without encapsulated or surface coupled proteins, selectively binds to the PEG on a second dose of the 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. Although further detailed study is required to define the exact mechanism for the phenomenon, our earlier results obviously demonstrate that "empty" PEGylated liposomes can induce responses of the immune system.

It is well known that T cell-independent (TI) response, characterized by a rapid production of low affinity polyclonal IgM, is one of the natural defense mechanisms of the host to remove invading antigens [14,15]. Generally, the characteristics of TI antigen are large molecular weights, highly repetitive structures and poor in vivo degradability [15]. Current PEGylated liposomes greatly differ from the earlier haptenized liposomes, categorized as a TI antigen, in terms of lipid composition, size and surface-modification. However, PEGylated liposomes may function as a TI antigen because surface modified PEG has characteristics similar to TI antigens and lacks peptide components that can be presented to helper T cells to initiate an immune response.

Earlier studies clearly indicated that the immune response, anti-PEG IgM production, is initiated by intravenous injection of "empty" PEGylated liposomes under certain conditions [13,16]. Besides the haptenized multilamellar liposomes [17,18], Li et al. [19] have recently reported that TI immune responses were enhanced by incorporating PEGylated-lipid derivative in CpG-oligodeoxynucleotide (ODN) encapsulated liposomes. However, to date, there has been no report showing that "empty" PEGylated liposomes cause TI immune response following intravenous injection. In this study, therefore, the issue of whether "empty" PEGylated liposomescan initiate the immune response against PEG as a TI antigen was studied.

2. Materials and methods

2.1. Animal

Male Wistar rats (250–300 g) and male BALB/c Slc-nu/nu (20–25 g) mice were purchased from Japan SLC (Shizuoka, Japan). Upon arrival, animals were allowed to acclimatize for at least one week. Animals had free access to water and chow, and were housed under controlled environmental conditions (constant temperature, humidity, and 12 h dark–light cycle). Mice were maintained under pathogen-free conditions. All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

2.2. Reagents and antigens

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-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. All other reagents were of analytical grade. Lipopolysaccharide (LPS) (LPS from *E. coli* serotype 0111:B4, phenol extraction) and cyclophosphamide monohydrate were purchased from Sigma (MO, USA). 2,4,6-trinitrophenyl-aminoethylcarboxymethyl (TNP)-LPS, TNP-Ficolland TNP-BSA were purchased from Biosearch Technologies (CA, USA).

2.3. Preparation of liposome

PEGylated liposome, composed of HEPC: CHOL: $mPEG_{2000}$-DSPE (1.85:1.0:0.15 molar ratio), was prepared as previously described [7]. 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). The liposomes were sized by subsequent extrusion through polycarbonate membrane filters (Nuclepore, CA., USA) with pore sizes of 400 (×3), 200 (×3), 100 (×5) and 80 (×3) nm. The mean diameters of the prepared liposomes were determined at room temperature by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA) and found to be 112±14 nm. The concentration of phospholipid was determined by a colorimetric assay [20].

2.4. Detection of IgM or IgG response against PEG

A simple ELISA procedure was employed to detect IgM and IgG against PEG based on a method described for the detection of anti-CHOL antibody (Ab) [21] using the Rat IgM ELISA Quantification Kit, the Rat IgG ELISA Quantification Kit and the Mouse IgM ELISA Quantification Kit (Bethyl Laboratories, TX, USA). Briefly, 10 nmol of $mPEG_{2000}$-DSPE in 50 μl 100% ethanol was added to 96-well plates. Lipid-coated plates were allowed to air dry completely for 2 h before being blocked for 60 min with Tris-buffered saline (50 mM Tris, 0.14 M NaCl, pH 8.0) containing 1% BSA and subsequently washed 3 times with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20. Diluted serum samples (1:500) (100 μl) were then applied in appointed wells for 1 h and washed 5 times as before. Horseradish peroxidase (HRP)-conjugated Ab (100 μl) 1 μg/ml (Goat anti-Rat IgM IgG-HRP conjugate, Goat anti-Rat IgG IgG-HRP conjugate or Goat anti-mouse IgM-HRP conjugate) in the conjugate diluent (Tris-buffered saline (pH 8.0) containing 1% BSA and 0.05% Tween 20) was added to each well. After incubation for 60 min, the wells were washed five times with the wash solution. The coloration was initiated by adding 100 μl of *o*-phenylene diamine (1 mg/ml) (Sigma, MO, USA). After 15 min incubation, the reaction was stopped by adding 100 μl of 2 N-H_2SO_4 and the absorbance was measured at 490 nm using a Microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were performed at room temperature.

2.5. Depletion of marginal zone (MZ) lymphocytes by intraperitoneal injection of cyclophosphamide

Depletion of splenic MZ lymphocytes with cyclophosphamide in rats was carried out according to the method previously

described [22]. Cyclophosphamide, freshly dissolved in sterile distilled water at 5 mg/ml, was injected intraperitoneally at a dose of 400 mg/m^2. Instead of cyclophosphamide solution, HEPES buffered saline (pH 7.4) was injected intraperitoneally as a control. Eight days later, PEGylated liposomes (0.001 μmol phospholipid/kg), TNP-LPS (100 μg/kg) or TNP-BSA (5 mg/kg) was intravenously injected into those rats. Then, 5 days later, blood was withdrawn and serum was collected from the rats. The IgM response against PEG on PEGylated liposomes and TNP on LPS or BSA was determined using the Rat IgM ELISA Quantification Kit. A mPEG$_{2000}$-DSPE-coated plate was prepared as described above. TNP-BSA was coated on a 96-well plate by incubation of TNP-BSA solution (10 μg/ml) for 2 h. Those plates were then blocked for 60 min with Tris-buffered saline (pH 8.0) containing 1% BSA and subsequently washed 3 times with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20. Diluted serum samples (1:500) (100 μl) were then applied in appointed wells and incubated for 1 h, and then washed 5 times as before. HRP-conjugated Ab (100 μl) 1 μg/ml (Goat anti-Rat IgM IgG-HRP conjugate) in the conjugate diluent (Tris-buffered saline (pH 8.0) containing 1% BSA and 0.05% Tween 20) was added to each well. After incubation for 60 min, the wells were washed five times with the wash solution. The coloration was initiated by adding 100 μl of OPD solution (1 mg/ml). 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). All incubations were performed at room temperature.

2.6. In vitro lymphocyte proliferation

The spleen was removed from a rat under anaesthetization. Spleen cell suspension was prepared as described previously [23]. Briefly, spleen slices were pressed through a Cell Strainer (100 m, Becton Dickinson, NJ, USA). The cells were suspended in RPMI1640 (Nissui, Japan) supplemented with 100 units/ml penicillin, 100 units/ml streptomycin and 10% heat-inactivated fetal bovine serum (Sigma, MO, USA). Red blood cells were lysed by treatment with ammonium chloride lysis buffer (0.83% NH_4CL) for 5 min on ice. Cells were washed twice with RPMI1640 and filtered to remove clumps using a Cell Strainer.

Lymphocyte proliferation was evaluated by BrdU Cell Proliferation Assay Kit (Exalpha Biologicals, Boston, USA) according to the method recommended. In brief, spleen cells were seeded at 2×10^4 cells in 100 μl of complete culture medium into a 96-well culture plate. The test reagents (100 μl) ((PEGylated liposomes (0.00001, 0.01, 1, 100 μM), TNP-Ficoll (0.005, 0.05, 0.5, 5 mg/ml), TNP-LPS (0.005, 0.05, 0.5, 5 mg/ml) or sterile HEPES buffered saline (control))were added to each well and then incubated for 48 h in a humidified atmosphere (37 °C, 5% CO_2). Then, 20 μl of BrdU solution was added to the appropriate wells and incubated for another 24 h. After centrifuging the plates for 5 min at 1000 rpm, the medium was removed by aspiration. The fixing-denature solution (200 μl) was added into each well and then incubated for 30 min. BrdU label was detected by the anti-BrdU monoclonal Ab. The mouse anti-BrdU monoclonal detector Ab was diluted at 1:200 with Ab diluent. After the washing step, the secondary HRP-conjugated goat anti-mouse IgG, diluted at 1:2000 with Ab diluent, was added. After 30 min incubation, the coloration was initiated by adding tetra-methylbenzidine. After 15 min incubation, the reaction was stopped by adding 100 μl of 2 N H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader (Wallac1420 ARVOsx). All incubations were performed at room temperature.

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. Anti-PEG IgG or IgM response in rats after intravenous injection of "empty" PEGylated liposomes

This group recently reported that a single first dose of "empty" PEGylated liposomes is capable of inducing a strong anti-PEG IgM response [16]. But, the issue of whether a primary anti-PEG IgG response is also induced by injection of the PEGylated liposomes was not studied. The first investigation studied the primary IgG or IgM response against PEG in rats following intravenous injection of "empty" PEGylated liposomes at a dose of 0.001 μmol phospholipid/kg, which is capable of causing the ABC phenomenon at a maximum level [13,24]. Simultaneous IgM and IgG responses against PEG were observed at 3–5 days for IgG and 3–10 days for IgM after the first injection (Fig. 1). The IgM responses were relatively much higher than IgG responses. IgM level against PEG began

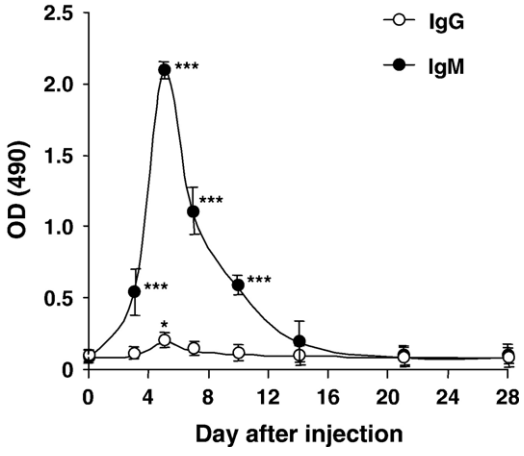

Fig. 1. Anti-PEG IgG and IgM responses following a single intravenous injection of PEGylated liposomes in rats. PEGylated liposomes were intravenously injected into rats at a dose of 0.001 μmol phospholipids/kg. Blood was withdrawn on days 3, 5, 7, 10, 14, 21 and 28 after the injection. The blood collected from naïve rats was used as a control. Anti-PEG IgM (●) and IgG (○) was detected with ELISA as described in 2.4. Each value represents the mean ± S.D. of three separate experiments. *$p<0.05$, ***$p<0.005$.

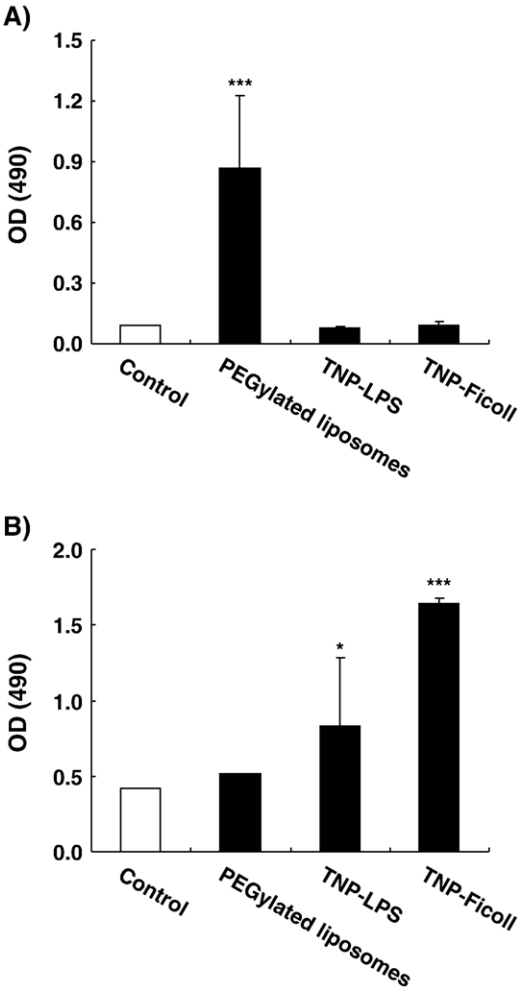

Fig. 2. Anti-PEG IgM response following intravenous injection of PEGylated liposomes into immunodeficient athymic (nude) BALB/c mice. PEGylated liposomes (25 μmol phospholipids/kg), TNP-LPS (2.5 mg/kg), TNP-Ficoll (2.5 mg/kg) or HEPES buffered saline (control) was intravenously injected into the immunodeficient athymic (nude) BALB/c mice. Ten days later, blood was withdrawn and serum was collected. The serum was divided into two. Anti-PEG IgM (A) and anti-TNP IgM (B) were determined with ELISA as described in 2.5. Each value represents the mean ± S.D. of three separate experiments. p value applies to differences between the control and PEGylated liposome-treatment. *$p<0.05$, ***$p<0.005$.

to increase at day 3, reached the maximum at day 5, then decreased gradually and returned to the basal level at day 14 after the first injection. A weak increase of IgG response was observed at day 5 and gradually disappeared by 10 days after the injection.

3.2. IgM response in nude mice following intravenous injection of "empty" PEGylated liposomes

To investigate whether "empty" PEGylated liposomes can initiate anti-PEG IgM response in the absence of T cells, PEGylated liposomes (25 μmol phospholipids/kg) were intravenously injected into immunodeficient athymic (nude) BALB/c mice. Anti-PEG IgM in blood was assessed on day 10 after the first injection (because the second dose of PEGylated liposomes was most rapidly cleared at day 10 after the first injection of 25 μmol phospholipids/kg in mice) [8]). The injection of PEGylated liposomes induced a more dominant IgM response than IgG (data not shown). Induced anti-PEG IgM level was much higher than that in control mice (Fig. 2A).

Additionally, the issue of whether immunodeficient athymic (nude) mice elicit responses to TNP-haptenized TI antigens (TNP-LPS, a TI-1 antigen, and TNP-Ficoll, a TI-2 antigen [25–27]) was evaluated. Injection of either TNP-LPS or TNP-Ficoll elicited anti-TNP IgM responses, whereas the injection did not elicit any anti-PEG IgM responses (Fig. 2B). Injection of "empty" PEGylated liposomes did not induce any anti-TNP

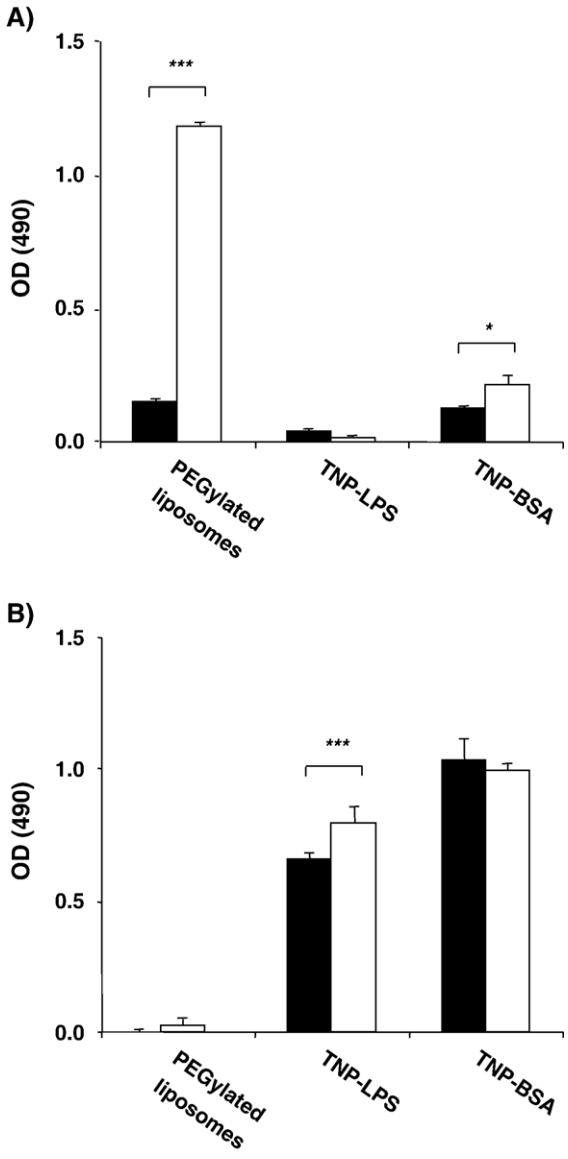

Fig. 3. Effect of depletion of MZ B-cell by intraperitoneal administration of cyclophosphamide on IgM response against PEG. Rats were treated with an intraperitoneal administration of cyclophosphamide (400 mg/m^2) (closed column) or HEPES buffered saline (open column) 8 days before PEGylated liposomes, TNP-LPS or TNP-BSA was intravenously injected. 5 days after intravenous injection, blood was withdrawn from the rats and the IgM against PEG and TNP in the blood was determined with ELISA as described in 2.5. (A) IgM response against PEG. (B) IgM response against TNP. Each value represents the mean ± S.D. of three separate experiments. *$p<0.05$, ***$p<0.005$.

response (Fig. 2B). These results clearly demonstrated that "empty" PEGylated liposomes induced an anti-PEG IgM response in a T cell-independent manner.

3.3. Effect of depletion of marginal zone (MZ) lymphocytes on the anti-PEG IgM response

The influence of the depletion of MZ lymphocytes on the IgM response against PEG or TNP following an intravenous injection of either "empty" PEGylated liposomes, TNP-LPS or TNP-BSA was investigated. Treatment with cyclophosphamide significantly suppressed the anti-PEG IgM response (Fig. 3A). Interestingly, TNP-BSA slightly induced an anti-PEG IgM response, whereas TNP-LPS did not induce such a response. In addition, the treatment with cyclophosphamide significantly suppressed the slightly induced anti-PEG IgM response (Fig. 3B). In contrast, "empty" PEGylated liposomes did not induce any anti-TNP IgM response. On the other hand, both TNP-BSA and TNP-LPS significantly induced an anti-TNP IgM response. Only the response against TNP-LPS was slightly suppressed by treatment with cyclophosphamide.

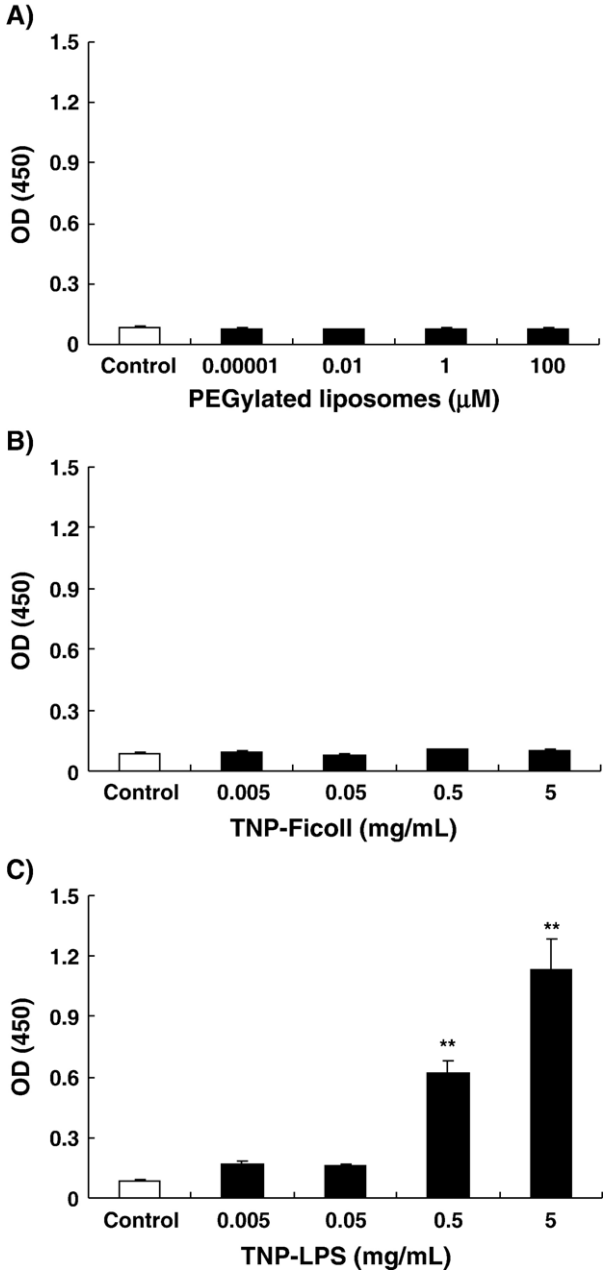

Fig. 4. In vitro splenic lymphocyte proliferation by stimulation with PEGylated liposomes, TNP-Ficoll and TNP-LPS. Splenic lymphocytes were incubated with (A) PEGylated liposomes (0.00001, 0.01, 1, 100 μM), (B) TNP-Ficoll (0.005, 0.05, 0.5, 5 mg/ml), (C) TNP-LPS (0.005, 0.05, 0.5, 5 mg/ml) or HEPES buffered saline (pH 7.4) (control). The lymphocyte proliferation was assessed by BrdU cell proliferation assay. Each value represents the mean ± S.D. of three separate experiments. p values apply to differences from the control. $^{**}p<0.01$.

3.4. In vitro splenic lymphocyte-proliferation assay by incubation with either "empty" PEGylated liposomes, TNP-LPS or TNP-BSA

Lymphocytes collected from the spleens of naïve rats were incubated with either "empty" PEGylated liposomes, TNP-Ficoll or TNP-LPS. Following incubation with TNP-LPS, a massive incorporation of BrdU into lymphocytes was detected at concentration of 0.5 to 5 mg/ml (Fig. 4C). In contrast, no significant BrdU incorporation was detected following incubation with TNP-Ficoll (0.005 to 5 mg/ml) (Fig. 4B) and "empty" PEGylated liposomes (0.00001 to 100 μM) (Fig. 4A). These results may suggest that "empty" PEGylated liposomes elicit an anti-PEG IgM response, as TI-2 antigen promotes a specific IgM response.

4. Discussion

PEGylated liposomes, designed as a drug delivery carrier and developed to avoid detection by the host defense system, are generally believed to be non-toxic and non-immunogenic. However, this group recently reported that "empty" PEGylated liposomes elicit anti-PEG IgM, which is responsible for rapid clearance of a second dose of PEGylated liposomes in rats [13]. This suggests that an unexpected immune reaction occurred upon intravenous injection of "empty" PEGylated liposomes. Furthermore, in this study, it was demonstrated that the anti-PEG IgM production following intravenous injection of "empty" PEGylated liposomes was detected in immunodeficient athymic (nude) BALB/c mice (Fig. 2A), which are employed to distinguish T cell-dependency or independency of the immune reaction [14,15,28]. It was also demonstrated that depletion of MZ lymphocytes (presumably B cells) in the spleen by treatment with cyclophosphamide [22] significantly suppressed anti-PEG IgM production (Fig. 3A). Taken together with our earlier report [12], these findings strongly suggest that B cells, not T cells, in the spleen are critical in the development of an immune response against "empty" PEGylated liposomes, and consequently induce the accelerated blood clearance (ABC) phenomenon.

As shown in Fig. 1, a single injection of "empty" PEGylated liposomes simultaneously induced both IgM and IgG responses, although the level of the IgG response was much lower than that of the IgM response. The pattern of immune response differed from the classic primary response against T cell-dependent (TD)

antigens in that the appearance of IgM was always earlier than that of IgG. On the contrary, the predominant response of IgM was consistent with the T cell-independent (TI) immune response, in that immunoglobulin induced by TI antigens usually remains IgM without T-cell help. This leads to a possible concern that "empty" PEGylated liposomes function as one of the TI antigens.

TI antigens are generally divided into type 1 (TI-1) and type 2 (TI-2), based on immunogenicity in nude mice and xid-mice [14,15,29]. Different subsets of TI antigen have different properties such as polyclonal B-cell activation, requirement of repetitive structures to induce the immune response. In this study, the in vitro stimulation by TNP-LPS, a typical TI-1 antigen, initiated significant proliferation of splenic lymphocytes as previously reported [30,31], while TNP-Ficoll, a typical TI-2 antigen, and "empty" PEGylated liposomes did not cause any proliferation in the range of lipid dose tested (Fig. 4). In earlier reports, repeating polymers such as polyvinylpyrrolidone and multilamellar liposomes have been introduced as the examples of TI-2 antigens [32]. Although further precise studies are required, it can be assumed that "empty" PEGylated liposomes (liposomes coated with repeating polymer-PEG) promotes immune reaction as TI-2 antigens do.

This group recently reported that the spleen plays an important role in the induction of IgM response after the injection of "empty" PEGylated liposomes [12]. It has been documented that intravenously injected long-circulating liposomes accumulated in the marginal zone of the spleen [33]. Demoy et al. [34] demonstrated that particles with different surface properties showed different localizations in spleen compartments. Therefore, it was proposed that the localization of PEGylated liposomes in a certain functional splenic compartment following intravenous injection and the subsequent interaction with the immune cells and/or accessory cells may be essential for the induction of an anti-PEG IgM response. Morphologically, the spleen can be divided into three major compartments; red pulp, white pulp and the marginal zone (MZ), which is defined as the junction of the red pulp and white pulp [35]. Among these compartments, the MZ was focused on because it contains macrophages, dendritic cells and B cells (MZ B-cell), and provides a first line of defense against blood borne pathogens [30]. Earlier and more recent results emphasized that MZ B cells generate a massive IgM response in humoral defense to TI, especially TI-2, antigens [36–38]. Kumararatne et al. [22] demonstrated that splenic MZ B cells, not follicular B cells, can be selectively depleted by a single intraperitoneal administration of cyclophosphamide, due to the slow renewal rate of the lymphocytes and the relatively susceptible locating microenvironment [39,40]. The depletion of MZ B cells in rats suppressed almost 90% of the anti-PEG IgM production (Fig. 3A). In contrast, the depletion of MZ B cells did not affect the immune response against hapten (TNP) derived by a TD antigen (TNP-BSA) [41] and only slightly affected that by a TI-1 antigen (TNP-LPS) [39] (Fig. 3B).These results suggest that MZ B cells are the 'ringleader' in response to the first dose of "empty" PEGylated liposomes, and that PEGylated liposomes promote the immune reaction against PEG as TI-2 antigens do. Consequently the ABC phenomenon is induced.

To date, there is no report demonstrating that PEGylated liposomes abrogate the immunological response in clinical settings when the liposomes are repeatedly administered to humans. However, several recent studies in animal experiments have demonstrated that innate and adaptive immune responses can be enhanced through encapsulation of DNA and ODN in lipid-based delivery vehicles [42–45], increasing the importance of evaluating carrier-directed effects on the immune system. Therefore, this study points out the potential occurrence of unexpected immune reactions upon intravenous injection of PEGylated liposomes or other PEGylated particles, and by extension, PEGylated proteins or nucleic acids.

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