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Transport of PEGylated liposomes from the splenic marginal zone to the follicle in the induction phase of the accelerated blood clearance phenomenon

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ABSTRACT

The accelerated blood clearance (ABC) phenomenon has been reported to enhance the clearance of PEGylated liposomes from the blood circulation when the liposomes are injected into the same animal repeatedly. We have shown that anti-PEG IgM production from splenic B cells is crucial in the ABC phenomenon. In this study, we describe the crucial role of marginal zone (MZ) B cells in the anti-PEG IgM production and recognition of PEGylated liposomes in the induction phase of ABC phenomenon. Suppression of the anti-PEG IgM production was correlated with the disappearance of IgM^{high} cells in the MZ, particularly MZ-B cells, following cyclophosphamide (CPA)-treatment, confirming that splenic MZ-B cells are responsible for anti-PEG IgM production. The MZ-B cells stimulated by a first dose of PEGylated liposomes internalized the second dose of PEGylated liposomes in a PEG modification-dependent manner and transported the liposomes into the follicle (FO) region. To the best of our knowledge, this is the first report showing that PEGylated liposome is recognized by MZ-B cells and transported to the FO region like blood-borne antigens or immune complexes. It is likely that PEGylated liposomes are recognized as a TI-2 antigen by the first line of defense against life-threatening infections by blood-borne organisms. Our study may have implications for immunogenicity of synthesized polymer-grafted therapeutics including nanocarriers, nucleic acids and proteins.

Introduction

It is well recognized that the presence of polyethylene glycol (PEG) on the surface of liposomes attracts a water shell, providing a steric barrier against plasma proteins or opsonins (Lasic et al. 1991; Senior et al. 1991; Torchilin et al. 1994). This, in turn, results in a decrease in both the rate and extent of uptake of liposomes by the cells of the mononuclear phagocyte system (MPS). Finally, intravenously injected PEGylated liposomes exhibit long circulating properties. Such PEGylated liposomes have been widely used as a carrier in drug delivery to improve the lifetime of encapsulated therapeutic agents (Allen et al. 1991; Klibanov et al. 1990; Papahadjopoulos et al. 1991).

Despite this defined concept, unexpected alterations in the pharmacokinetic behavior of PEGylated liposomes were observed when repeatedly injected at certain intervals into mice, rats or rhesus monkeys (referred to as the accelerated blood clearance (ABC) phenomenon) (Dams et al. 2000; Ishida et al. 2003; Laverman et al. 2001). An intravenous injection of PEGylated liposomes causes a second dose, injected a few days later, to lose its long-circulating characteristics and accumulate extensively in the liver. By further studies of our group, it was elucidated that anti-PEG IgM, produced from spleen in response to an injected first dose of PEGylated liposomes, is responsible for the alteration in the pharmacokinetics of a subsequent dose of PEGylated liposomes (Ishida et al. 2006b,c; Wang et al. 2007). Besides, the anti-PEG IgM production was detected in nude mice (Ishida et al. 2007), lacking T cells, but not in splenectomized mice (Ichihara et al. 2011) indicating that the anti-PEG IgM is secreted from splenic B cells without T-cell help. Furthermore, we observed that the level of anti-PEG IgM in serum began to increase at day 3, peaked at day 5, and then gradually decreased again (Ichihara et al. 2011; Ishida et al. 2007). It seems that two to three days are necessary for splenic B cells to mature and release anti-PEG IgM into the blood stream following injection of PEGylated liposomes.

The spleen is a crucial secondary lymphoid organ in the body's response against blood-borne antigens (Cyster 2000). The bulk of

Abbreviations: ABC, accelerated blood clearance; Chol, cholesterol; CPA, cyclophosphamide; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; FO, follicle; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; $mPEG_{2000}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (polyethylene glycol)-2000]; MPS, mononuclear phagocyte system; MZ, marginal zone; PE, phycoerythrin; PEG, polyethylene glycol; TI, T-cell independent; TI-1, T-cell independent type 1; TI-2, T-cell independent type 2.

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the spleen is composed of red pulp, which is the site of red blood cell disposal. The lymphocytes surround the arterioles entering the organ, forming areas of white pulp. The marginal zone (MZ) of the spleen is located at the border between the white pulp and red pulp, and outside the marginal sinus. Much of the antigen entering the spleen is retained at the marginal sinus, from where it can percolate through the marginal zone into the red pulp. The architectural structure of the MZ results in a strongly reduced blood-flow allowing intimate contact between antigens and effector cells (Harms et al. 1996). Thus, B cells in the MZ (MZ-B cells) are easily and continuously exposed to blood as opposed to B cells in the follicles (follicular B (FO-B) cells) and extensively contribute to a rapid first line of defense able to produce large amounts of specific IgM within 3–4 days after antigen stimulation (Martin and Kearney 2001; Zandvoort and Timens 2002). We therefore assumed that among the splenic B cells the MZ-B cells are responsible for the anti-PEG IgM production in the ABC phenomenon. However, so far, direct evidence showing the contribution of MZ-B cells to the anti-PEG IgM response was lacking. In addition, we had not yet elucidated how the MZ-B cells are activated by the first dose of PEGylated liposomes.

In this study, we investigated the involvement of MZ-B cells in anti-PEG IgM production by depletion of MZ-B cells by means of an intravenous injection of cyclophosphamide. In addition, we studied the behavior of the MZ-B cells in the spleen after the first dose of PEGylated liposomes with fluorescently labeled PEGylated test liposomes as an marker of activated, anti-PEG IgM over-expressing, MZ-B cells.

Materials and methods

Materials

Hydrogenated egg phosphatidylcholine (HEPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Chol) was purchased from Wako Pure Chemical (Osaka, Japan) and cyclophosphamide monophosphate (CPA) was purchased from Sigma (MO, USA). DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and DiO (3,3′-dioctadecyloxacarbocyanine perchlorate) were purchased from Invitrogen (Paisley, UK). All other reagents were of analytical grade.

Animals

Male Wistar rats (250–300 g) were purchased from Japan SLC (Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

Preparation of liposomes

PEGylated liposomes composed of HEPC:$mPEG_{2000}$-DSPE:Chol (1.85:0.15:1 molar ratio) and non-PEGylated conventional liposomes composed of HEPC:Chol (2:1 molar ratio) were prepared as described previously (Ishida et al. 2003). To detect the liposome distribution in spleen and their association with spleen cells, the liposomes were labeled with the hydrophobic fluorescent dye DiI or DiO (1 mol% of liposomal phospholipids). The mean diameter of the resulting liposomes was determined by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA), and was 102 ± 12 nm for PEGylated liposomes and 109 ± 15 nm for conventional liposomes, respectively. Phospholipid concentration in the liposome preparations was determined by colorimetric assay (Bartlett 1959).

ELISA for detection of anti-PEG IgM

A simple ELISA as described previously was employed to detect anti-PEG IgM (Ishida et al. 2007). Briefly, 10 nmol of PEG_{2000}-DSPE in 50 μl ethanol was added to each well of a 96-well plate. The plate was allowed to air dry completely for 2 h. The lipid-coated plates were then blocked for 1 h with Tris-buffered saline (pH 8.0) containing 1% BSA and were subsequently washed three times with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20 (wash solution). Diluted serum samples (1:100) (100 μl) were then applied to the wells, incubated for 1 h and washed five times with wash solution. Horseradish peroxidase (HRP)-conjugated antibody (100 μl, 1 μg/ml, Goat anti-rat IgM-HRP conjugate; Bethyl Laboratories, TX, USA) was added to the wells. After 1 h incubation, the wells were washed five times with wash solution. The coloration was initiated by adding 100 μl of *o*-phenylenediamine (1 mg/ml) (Sigma, MO, USA). After a 15-min incubation, the reaction was stopped by adding 100 μl of 2 M H_2SO_4. The absorbance was measured at 490 nm using a microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science). All incubations were performed at room temperature.

Depletion of MZ-B cells by CPA treatment

Depletion of splenic MZ-B cells with CPA was carried out according to a previously described method (Zandvoort et al. 2001). CPA, freshly dissolved in sterile saline at 20 mg/ml, was intravenously injected at a dose of either 30 or 40 mg/kg. Instead of CPA solution, saline was intravenously injected as a control. Eight days later, MZ-B cells in the spleen were detected by flow cytometry as described below.

Distribution of PEGylated liposomes in spleen

To evaluate the distribution of PEGylated liposomes in spleen, DiI-labeled PEGylated liposomes or DiO-labeled PEGylated liposomes (5 μmol/kg rat) were intravenously injected into rats. At 24 h post-injection, spleens were harvested and snap-frozen in optimal cutting temperature compound (Sakura Fintechnical, Tokyo, Japan) by dry-iced acetone. Sections of frozen samples (7 μm thick) were immediately examined using an AxioImager A1 (Zeiss, Oberkohen, Germany) and processed with Photoshop software (Adobe Systems, CA, USA). DiI was visualized using 549 nm excitation and 565 nm emission filter sets. DiO was visualized using 470 nm excitation and 525 nm emission filter sets.

Immunohistochemical analysis

For immunohistochemical analysis, after acquiring the image of liposome distribution in the frozen spleen section, frozen sections were subsequently fixed by acetone and were stained with FITC-labeled anti-rat IgM, or mouse anti-rat MZ-B (His57, Becton Dickinson, NJ, USA) and then phycoerythrin (PE)-labeled anti-mouse IgG (H&L, Rockland Immunochemicals, PA, USA). Images were acquired by AxioImager A1 and processed with Photoshop software.

Preparation of spleen cell suspensions and flow cytometry

Spleen cell suspensions were prepared as described previously (Ishida et al. 2007). Briefly, spleen slices were squeezed through a Cell Strainer (100 μm, Becton Dickinson, NJ, USA). The cells were suspended in PBS containing 0.5 mM EDTA (EDTA-PBS). Red blood