Intravenous Administration of Polyethylene Glycol-Coated (PEGylated) Proteins and PEGylated Adenovirus Elicits an Anti-PEG Immunoglobulin M Response

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A single intravenous administration of polyethylene glycol-coated (PEGylated) bovine serum albumin (BSA) and ovalbumin (OVA) elicited an anti-PEG immunoglobulin M (IgM) response, similar to that from PEGylated liposomes, although the administration did not elicit specific neutralizing antibodies to BSA and OVA. A cross-reactivity was observed between anti-PEG IgMs elicited by PEG-BSA and PEGylated liposomes. The anti-PEG IgM level induced by PEGylated proteins (BSA and OVA) reached the maximum at day 5 following intravenous injection. This production pattern was consistent with that induced by PEGylated liposomes. Splenectomy suppressed the anti-PEG IgM response against PEG-BSA and PEGylated liposomes. These observations relating PEG-BSA and PEGylated liposomes indicate that PEGylated proteins might promote the immune responses against PEG with a mechanism similar to that of PEGylated liposomes. In addition, a single intravenous administration of PEGylated adenovirus (PEG-Ad) also elicited an anti-PEG IgM response in a PEG-modification ratio dependent manner. To the best of our knowledge, this is the first report showing that an intravenous administration can elicit an anti-PEG IgM response against PEGylated substances. It appears that anti-PEG IgMs can be produced by the systemic administration of a PEGylated substance and may limit the efficacy of PEGylated substances such as proteins, Ad vector and nanoparticles, due to a cross-reactivity seen in some patients. The immunogenicity of PEGylated substances is usually tested against those very substances, rather than against covalently attached PEG. Our study suggests that the PEG immunogenicity of PEGylated therapeutic agents and particles merits further investigation.

Key words PEGylation; anti-polyethylene glycol immunoglobulin M; adenovirus; liposome

Biomedicines, such as protein drugs^{1,2)} and nucleic acid drugs,³⁾ have attracted considerable attention due to their high biological activity and specificity against target molecules. However, their application is sometimes limited due to their insufficient clinical effects as a result of susceptibility to destruction by proteolytic or nucleolytic enzymes, a short circulating half-life, low solubility, rapid kidney clearance and propensity to generate neutralizing antibodies.⁴⁾ Covalent conjugation of polyethylene glycol (PEG) to the biological active molecules, by a process called “PEGylation,” is one of the promising strategies being used to overcome these limitations.^{1–3)}

PEG is one of the most versatile synthetic polymers and is widely believed to lack immunogenicity and toxicity and shows high solubility in water and in many organic solvents.^{4,5)} For instance, proteins modified with PEG have an increased solubility due to the hydrophilicity of grafted PEG; their destruction by protease and the immune system is decreased due to the steric hindrance of grafted PEG; and, glomerular filtration is decreased due to an increase in the apparent size or hydrodynamic volume of a given protein.^{6–8)} Consequently, the pharmacokinetics of bioactive proteins is improved and thereby the PEGylated proteins become useful for therapy. At present, several PEGylated proteins, such as Adagen[®] (PEG adenosine deaminase), Oncaspar[®] (PEG-asparaginase), PegIntron[®] (PEG-interferon $\alpha 2b$), Pegasys[®] (PEG-interferon $\alpha 2a$), Neulasta[™] (PEG-G-CSF), Somavert[™]

(PEG-hGH), Mircera (PEG-erythropoietin), Cimzia (PEG-anti-tumor necrosis factor (TNF) α Fab) and one PEGylated oligonucleotide (Macuge[™], Branched PEG-anti-vascular endothelial growth factor (VEGF) aptamer) have been approved by Food and Drug Administration (FDA).^{9,10)} In addition, the PEGylation of a nanocarrier system, such as liposome, attracts a water shell, resulting in the reduced adsorption of opsonins and thus the recognition of the carrier by the cells of the mononuclear phagocyte system (MPS).¹¹⁾ The PEGylated nanocarriers thus obtained possess long circulation properties, and therefore can accumulate efficiently in solid tumors because of the enhanced vascular permeability and retention (EPR) effect in growing tumors.¹²⁾ Doxorubicin-encapsulated PEGylated liposome (Doxil[®] and Caelyx[®]) has been approved for use in the treatment of Kaposi's sarcoma and both ovarian and breast cancer.^{13,14)} Furthermore, although still in the pre-clinical stage, PEGylated adenoviruses (PEG-Ad) have been developed and have shown higher accumulation rates, stronger tumor-suppressive activity, and fewer hepatotoxic side effects than unmodified adenoviruses (Ad).^{10,15–18)}

In contrast to the preceding argument, we and others have reported that PEGylated liposomes lose the expected long circulating property when they are injected repeatedly in the same animal (the so-called accelerated blood clearance (ABC) phenomenon).^{19,20)} On the basis of our earlier results,^{21,22)} we proposed the following tentative mechanism for the cause of this phenomenon: anti-PEG IgM, which is produced in the spleen in response to the initial dose, selectively binds to the PEG of the second dose liposomes injected several days later

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and subsequently activates the complement system, and, as a consequence, the liposomes are taken up by the Kupffer cells in the liver. Although it is widely accepted that PEG is non-immunogenic, our studies suggest the possibility that PEG on the macromolecule acquires immunogenicity and elicits anti-PEG IgM. However, there has been no cross-sectional research on anti-PEG IgM production among different PEGylated substances or particles.

In the present study, therefore, we examined the simple issue of whether PEGylated proteins and Ad elicit an anti-PEG IgM response, as PEGylated liposome do. Herein, we show that both PEGylated proteins and PEGylated Ad elicit an anti-PEG IgM following a single intravenous injection. To the best of our knowledge, this is the first report regarding the immunogenicity of PEG that has been covalently attached to proteins and Ad following intravenous administration.

MATERIALS AND METHODS

Materials Methoxypolyethylene glycol succinimidyl glutarate-2000 or 30000 (mPEG₂₀₀₀-SGA or mPEG₃₀₀₀₀-SGA), methoxypolyethylene glycol succinimidyl propionate-5000 or 20000 (mPEG₅₀₀₀-SPA or mPEG₂₀₀₀₀-SPA), hydrogenated egg phosphatidylcholine (HEPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*n*-methoxy (polyethylene glycol)-2000 (mPEG₂₀₀₀-DSPE) were generously donated by NOF (Tokyo, Japan). Cholesterol (Chol) was of analytical grade (Wako Pure Chemical Ind., Ltd., Osaka, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

Preparation of PEGylated Liposome PEGylated liposome, composed of HEPC:Chol:mPEG₂₀₀₀-DSPE=1.85:1.0:0.15 (molar ratio), was prepared as previously described.²⁰ Briefly, the lipids were dissolved in chloroform, and after evaporation of the organic solvent, the resultant lipid film was hydrated in *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4). The liposome was sized by subsequent extrusion through polycarbonate membrane filters (Nuclepore, CA, U.S.A.) with pore sizes of 400, 200, 100, and 80 nm. The mean diameter of the prepared liposome was determined by using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, U.S.A.) and was 113±19 nm. The concentration of phospholipids was determined by colorimetric assay.²³

Preparation of PEGylated Proteins PEGylated proteins were prepared by adding a 4–50 molar excess of activated PEG to bovine serum albumin (BSA) or ovalbumin (OVA) with gentle stirring according to a previously reported method.^{8,9} BSA was reacted with PEG₂₀₀₀-SGA or PEG₃₀₀₀₀-SGA at 37°C for 1 h. OVA was reacted with PEG₅₀₀₀-SPA or PEG₂₀₀₀₀-SPA at 37°C for 30 min. To eliminate unreacted PEG and protein, the reaction mixture was applied to a Sephacryl S-400HR (PEG-BSAs) or Superose 12–10/300 GL (PEG-OVAs) (GE healthcare, Chalfont, U.K.) gel filtration column. The fractions containing PEGylated protein were collected and concentrated using an Amicon Ultra centrifugal filter device (Millipore Corp, Bedford, MA, U.S.A.). The approximate molecular weight of each PEGylated protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to a previously reported method.⁸ PEG₂₀₀₀-BSA was 116–200 kDa; PEG₃₀₀₀₀-BSA was

>200 kDa; PEG₅₀₀₀-OVA was 66–97 kDa; and, PEG₂₀₀₀₀-OVA was >200 kDa.

Preparation of PEGylated Adenoviruses (Ads) We employed an E1-E3-deleted AD type 5 vector constructed by an improved *in vitro* ligation method.^{24,25} PEGylation to the Ad vector was carried out according to a previously demonstrated method.¹⁷ Briefly, Ad was incubated with mPEG₅₀₀₀-SPA or mPEG₂₀₀₀₀-SPA at molar excesses of 50-, 200-, 800-fold at 37°C for 45 min with gentle stirring to allow modification of the viral lysine residues. The modification ratio of each PEGylated adenovirus (PEG-Ad) was determined by SDS-PAGE analysis as described previously.^{15,16} The ratios were approximately 30, 45 and 90%, and were dependent on the PEG amount required to elicit a reaction. The particle size of PEG-Ad was measured using a ZETASIZER 3000HS (Malvern, U.K.) and was approximately 100 nm, irrespective of the modification ratio and size of the PEG.

Animal Experiment 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. PEGylated liposome, PEGylated protein (PEG-BSA and PEG-OVA) and PEG-Ad were intravenously injected into rats at a dose of 0.0002 μmol phospholipids/animal, 1 μg protein/animal, and 10¹¹ particles/animal, respectively. At indicated days after administration, blood samples were collected from each rat. To obtain serum, the blood was centrifuged at 3000 rpm and 4°C for 15 min. To study the effect of the spleen on the anti-PEG IgM response against PEGylated materials, a splenectomy (removal of spleen) was performed as previously described.²¹ The level of anti-PEG IgM in serum was determined by enzyme-linked immunosorbent assay as described below.

Enzyme-Linked Immunosorbent Assay Each well of the 96-well microtiter plate was coated with 10 nmol of PEG-DSPE or 1 μg of PEG-BSA by incubation for 2 h. The coated plate was washed 3 times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween20). Then blocking buffer (50 mM Tris, 0.14 M NaCl, 1% BSA) was added and incubated for 1 h. After 3 cycles with the wash solution, the diluted sample sera in the sample buffer (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween20) were added and incubated for 1 h. Then, the plate was washed 5 times with wash solution. Horseradish peroxidase-conjugated antibody (goat anti-mouse immunoglobulin M (IgM) IgG-horseradish peroxidase (HRP) conjugate; Bethyl Laboratories, TX, U.S.A.) was added and incubated for a further 1 h. The plate was washed 5 times with wash solution, and then incubated for 5–15 min followed by the addition of *o*-phenylene diamine (Sigma, MO, U.S.A.). The reaction was stopped by adding 2 M H₂SO₄. Absorbance at 490 nm was measured with a Microplate reader (Wallac1420 ARVOsx, PerkinElmer Life Science).

CH₅₀ Measurement To determine whether the PEGylated materials activate the serum complement system, the residual serum complement activity was measured by functional complement assay (CH₅₀) following the incubation of serum with various PEGylated materials. CH₅₀ was determined according to the method described previously²² with sheep red blood cells sensitized with monoclonal anti-sheep IgM antibody (SRBC) (CH₅₀ SEIKEN, DENKA SEIKEN, Tokyo, Japan). Briefly, 90 μL of rat serum was collected from

Fig. 1. Anti-PEG IgM Response to PEGylated Proteins

PEGylated proteins ($1\mu\text{g}/\text{rat}$) were intravenously injected into rats. At day 3, 5, 7, and 10, sera were collected from the rats, and anti-PEG IgM in the sera was determined by ELISA using mPEG₂₀₀₀-DSPE coated 96-well plates. Positive control represents the anti-PEG IgM level elicited by intravenous injection of PEGylated liposome ($0.0002\mu\text{mol}$ phospholipids/animal) at day 5. Each value represents the mean $\pm$ S.D. ($n=3$). Open circle, PEG₂₀₀₀-BSA; Closed circle, PEG₃₀₀₀₀-BSA; Open square, PEG₅₀₀₀-OVA; Closed square, PEG₂₀₀₀₀-OVA. * $p<0.05$ and ** $p<0.01$ vs. the titer of anti-PEG IgM at day 0.

rats that had received an intravenous injection of PEG-BSAs ($1\mu\text{g}$ protein/animal), PEGylated liposome ($0.0002\mu\text{mol}$ phospholipids/animal) or HEPES. The serum then was incubated with PEGylated liposome or PEG-BSA at 37°C for 15 min. Then, $10\mu\text{L}$ aliquots of the treated serum was diluted with 1 mL of veronal-buffered saline (VBS; 150mM NaCl, 75mM sodium barbital, 0.15mM CaCl_2 , 1mM MgCl_2 , 5% D-glucose, 0.1% gelatin (DGV B^{2+}). Then, 0.4mL of SRBC ($5\times 10^8/\text{mL}$) was mixed with 2.6mL of sequentially further diluted PEGylated materials-treated serum and incubated at 37°C for 1 h. Unlysed SRBC were pelleted by centrifugation (2000rpm , 10 min, 4°C) and the amount of hemoglobin released into the supernatant was quantified spectrophotometrically at 541nm (UV-1600, Shimadzu, Kyoto, Japan). The CH_{50} was calculated according to the equations recommended by the manufacturer. The residual complement activity was calculated by dividing the CH_{50} value in the liposome-treated sample by that in naïve serum.

Statistics All mean values are expressed as the mean $\pm$ S.D. Statistical analysis was performed with a two-tailed unpaired t -test and a one-way analysis of variance (ANOVA) using GraphPad InStat software (GraphPad Software, CA, U.S.A.). The level of significance was set at $p<0.05$.

RESULTS

Anti-PEG IgM Response to PEGylated Proteins Following a single intravenous injection of either PEG₂₀₀₀-BSA, PEG₃₀₀₀₀-BSA, PEG₅₀₀₀-OVA or PEG₂₀₀₀₀-OVA, serum anti-PEG IgM level was determined with a mPEG₂₀₀₀-DSPE coating plate at indicated time points. As shown in Fig. 1, regardless of the type of protein and chain length of modified PEG, all PEGylated proteins induced anti-PEG IgM production. The serum anti-PEG IgM level began to increase at day 3, reached the maximum at day 5, and then decreased gradually. The level of anti-PEG IgM response was correlated to the length of the PEG chain attached to proteins under our experimental

Fig. 2. Anti-PEG IgM Response to PEG-BSAs in Splenectomized Rats

The spleen was removed from rats 7 d before intravenous injection of either PEG₂₀₀₀-BSA ($1\mu\text{g}/\text{animal}$), PEG₃₀₀₀₀-BSA ($1\mu\text{g}/\text{animal}$) or PEGylated liposome ($0.0002\mu\text{mol}$ phospholipids/animal). At day 5 after the injection, anti-PEG IgM in the serum was determined by ELISA using mPEG₂₀₀₀-DSPE-coated plate. Open column represents control (sham-operated) animal. Closed column represents splenectomized animal. Each value represents the mean $\pm$ S.D. ($n=3$). * $p<0.05$ and ** $p<0.01$ vs. the anti-PEG IgM production in non-splenectomized (sham-operated) rats.

Fig. 3. Cross-Reactivity of Anti-PEG IgM Induced by PEG-BSAs and PEGylated Liposome

The cross-reactivity of anti-PEG IgM induced by either PEG₂₀₀₀-BSA ($1\mu\text{g}/\text{animal}$), PEG₃₀₀₀₀-BSA ($1\mu\text{g}/\text{animal}$) or PEGylated liposome ($0.0002\mu\text{mol}$ phospholipids/animal) was determined by ELISA using 96-well plates coated with either BSA (open column), PEG₂₀₀₀-BSA (light gray column), PEG₃₀₀₀₀-BSA (dark gray column) or mPEG₂₀₀₀-DSPE (closed column). Each value represents the mean $\pm$ S.D. ($n=2-3$). * $p<0.05$ and ** $p<0.01$ vs. the OD in mPEG₂₀₀₀-DSPE coated wells.

condition.

Anti-PEG IgM Response in Splenectomized Rats To investigate the contribution of the spleen to the anti-PEG IgM response to PEGylated protein, a splenectomy (removal of spleen) was carried out 7 d before the intravenous injection of either PEG-BSAs (PEG₂₀₀₀-BSA and PEG₃₀₀₀₀-BSA) or PEGylated liposome (positive control). At day 5 after the injection, anti-PEG IgM in the serum was determined with a mPEG₂₀₀₀-DSPE coating plate (Fig. 2). Splenectomy suppressed anti-PEG IgM production against PEG₂₀₀₀-BSA and PEG₃₀₀₀₀-BSA as well as against PEGylated liposome. It appears that the spleen is the major organ that secretes anti-PEG IgM against PEG-BSAs (PEG₂₀₀₀-BSA and PEG₃₀₀₀₀-BSA).

Cross-Reaction of Anti-PEG IgM with PEGylated Liposome and PEGylated Protein To characterize anti-PEG

IgM induced by PEG-BSAs (PEG₂₀₀₀-BSA and PEG₃₀₀₀₀-BSA) and PEGylated liposome, the cross-reactivity of anti-PEG IgM induced by one of the PEGylated derivatives to each PEGylated derivative (PEG₂₀₀₀-BSA, PEG₃₀₀₀₀-BSA or mPEG₂₀₀₀-DSPE) (Fig. 3). Anti-PEG IgM elicited by PEG₂₀₀₀-BSA showed reactivity similar to PEG₂₀₀₀-BSA, PEG₃₀₀₀₀-BSA, and mPEG₂₀₀₀-DSPE. Anti-PEG IgM elicited by PEG₃₀₀₀₀-BSA also showed similar reactivity to PEG₂₀₀₀-BSA, PEG₃₀₀₀₀-BSA, and mPEG₂₀₀₀-DSPE. The anti-PEG IgM elicited by PEGylated liposome modified with mPEG₂₀₀₀-DSPE showed higher reactivity to mPEG₂₀₀₀-DSPE than to PEG-BSAs. Interestingly, PEG-BSAs induced no IgM response to BSA at day 5 after the intravenous injection of PEGylated BSA.

Anti-PEG IgM-Mediated Complement Activation The residual complement activity in the serum containing anti-PEG IgM was estimated as an indication of anti-PEG IgM-mediated complement activation by the PEGylated substances. The complement activity in the sera following incubation with various PEGylated substances (PEG₂₀₀₀-BSA, PEG₃₀₀₀₀-BSA and PEGylated liposome) was determined using a CH₅₀ assay kit (Fig. 4). The complement activity in the HEPES-treated sera from the rats that received each PEGylated material was set at 100%. No PEGylated substances consumed complement in the sera from the rats that had received HEPES. Also, in the sera from the rats that had received PEG₂₀₀₀-BSA, no consumption was induced by incubation with PEGylated substances. To the contrary, in the sera of the rats that had received PEG₃₀₀₀₀-BSA, significant consumption was induced by incubation with PEG₃₀₀₀₀-BSA and PEGylated liposome. Interestingly, in the sera from rats that had received PEGylated liposome, only PEGylated liposome consumed the complement, but other PEGylated substances were hardly consumed.

Anti-PEG IgM Response to PEGylated ADs In order to improve the resident time in the blood and attenuate immunogenicity, PEGylation has been preferentially attempted with a virus vector such as Ad.^{15–18} We investigated whether PEG-Ad elicits an anti-PEG IgM response similar to PEG-proteins and PEGylated liposome as described above. All PEG-Ads used in this study obviously induced an anti-PEG IgM response at day 5 after a single intravenous injection (Fig. 5). The PEG-modification ratio of Ad strongly affected the level of anti-PEG IgM production. Both PEG₅₀₀₀-Ad (45%) and PEG₂₀₀₀₀-Ad (45%) induced a significantly higher response than other PEG-Ads with different modification ratios (30% or 90%). The levels of anti-PEG IgM response elicited by PEG₅₀₀₀-Ad (45%) and PEG₂₀₀₀₀-Ad (45%) were comparable to that by PEGylated liposomes (positive control). It appeared that the chain length of PEG promotes the anti-PEG IgM response against PEG-Ad.

DISCUSSION

In the present study, we showed that PEG-proteins (PEG-BSA and PEG-OVA) and PEG-Ad induce an anti-PEG IgM response, as PEGylated liposomes do, following a single intravenous injection (Figs. 1, 5). This is a surprising result because PEG conjugates are widely regarded as having low immunogenicity and being immunologically safe.^{9,26} Hence, PEGylation technology successfully generated several FDA-approved proteinous and nucleic substances and nanocarriers that are considered non immunogenic, and the technology continuously applies to new substances such as nucleic acids,

Fig. 4. Anti-PEG IgM-Mediated Complements Activation

To obtain the sera containing anti-PEG IgM, PEG-BSAs or PEGylated liposome, each was intravenously injected into rats. At day 5, the sera were collected. The sera were incubated with HEPES (open column), PEG₂₀₀₀-BSA (light gray column), PEG₃₀₀₀₀-BSA (dark gray column) or PEGylated liposome (closed column) to consume the complement. Residual complement activity in the treated sera was assayed using a CH₅₀ kit. Each value represents the mean $\pm$ S.D. ($n=3$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. HEPES-treated sera (open column, negative control)

Fig. 5. Anti-PEG IgM Response to PEG-Ad

Various PEG-Ads (10^{11} particles/animal) were intravenously injected into rats. Five days later, anti-PEG IgM in the sera was determined by ELISA with mPEG₂₀₀₀-DSPE-coated 96-well plates. Positive control represents the amount of anti-PEG IgM elicited by PEGylated liposome ($0.0002 \mu\text{mol}$ phospholipids/animal). Each value represents the mean $\pm$ S.D. ($n=4$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. PBS

several nanocarriers and viral vectors.^{3,10} However, our results clearly indicate that the covalent coupling of PEG to substances results in either the loss or the masking of the original antigenicity and immunogenicity as expected, but also results in the appearance of the antigenicity and immunogenicity of PEG, neither of which has ever been shown by the intravenous injection of PEG itself.

There were several reports relating anti-PEG antibody responses against PEGylated proteins and PEG itself. It must be noted that such immunological responses were entirely promoted by subcutaneous administration, and not by intravenous administration. Richter and Akerblom²⁷ showed that the subcutaneous administration of PEG (5900 Da) to mice would elicit a weak and transitory immune response to PEG. They also showed a stronger anti-PEG immune response to PEG-OVA subcutaneously administered with Freund's complete

adjuvant. In addition, Ganson *et al.*²⁸⁾ reported that anti-PEG antibody was elicited in a phase I trial of subcutaneous PEGylated urate oxidase. A similar response to PEG has been observed in patients receiving intramuscular administration of FDA-approved Oncaspar® (PEG-asparaginase).²⁹⁾ In the current study, we showed that a single intravenous injection of PEG-proteins will elicit an anti-PEG IgM response (Fig. 1). This suggests that the intravenous administration of PEGylated proteins is clinically recommended, but it may induce an immunological response to PEG grafted to the proteinous subjects. To the best of our knowledge, this is the first report showing an immunological response to PEG following intravenous administration with PEGylated proteins.

In the present study, we employed BSA and OVA as test proteins which are exogenous proteins to mouse. There is a possibility that such exogenous proteins covalently attached to PEG might induce anti-PEG IgM response. Rubin³⁰⁾ have reported that haptenized endogenous protein induces anti-hapten antibody, as haptenized exogenous protein does. Klaus *et al.*³¹⁾ studied on the immunological properties of haptens coupled to thymus-independent (TI) carrier molecules and showed that the carrier need not be immunogenic for the induction of anti-hapten antibody responses. There seems no difference upon production of anti-hapten antibody such as anti-PEG response between exogenous proteins and endogenous proteins, although further study to prove must be required.

It is easy to be concerned that the presence of elicited anti-PEG IgM in blood circulation would affect the pharmacokinetics of a subsequent dose of PEGylated substances. We have previously shown that the anti-PEG IgM elicited by the initial dose of PEGylated liposome is responsible for an enhanced clearance of the subsequent dose as a result of complement activation.^{22,32)} A similar phenomenon was observed in PEG-modified poly lactide (PLA)-nanoparticles³³⁾ and in PEG-modified micelles.³⁴⁾ Roffler and co-workers^{35,36)} have generated anti-PEG IgM monoclonal antibody by immunization with an antibody-PEG-modified β -glucuronidase (β G) and have shown that when intravenously injected the generated anti-PEG IgM induced an accelerated clearance of PEG-modified β G from blood circulation. Our current study demonstrated that the elicited anti-PEG IgM has a cross-reactivity between PEG grafted onto proteins and PEG on nanoparticulate liposome (Fig. 3). In addition, PEG₃₀₀₀₀-BSA activated complement system presumably was mediated by anti-PEG IgM elicited by a previous dose (Fig. 4). Taken together, these results suggest that the administration of 1 PEGylated substance may accelerate the clearance of subsequent doses of the same PEGylated substance or another PEGylated substance, resulting in a reduced therapeutic efficacy of the PEGylated derivative. This would be another problem for PEGylation, which was originally expected to increase the residence time of the substance in the body.

It is hard to define the region of PEG that is recognized by an anti-PEG IgM. Richter and Akerblom²⁷⁾ proposed in an earlier study that the antigenic determinant of PEG may be a sequence of 6–7 $-\text{CH}_2\text{CH}_2\text{O}-$ units. Roffler and co-workers^{35,36)} demonstrated that the monoclonal anti-PEG IgM generated by antibody-PEG-modified β G recognizes the repeating sequence (16 $-\text{CH}_2\text{CH}_2\text{O}-$ units) of PEG. Thus, the anti-PEG IgM described in the present study may bind to the repeating sequence ($-\text{CH}_2\text{CH}_2\text{O}-$ units) of PEG.

Consistent with our earlier observations for PEGylated liposomes,³⁷⁾ following the intravenous injection with PEGylated proteins, anti-PEG IgM level began to increase at day 3, reached the maximum at day 5, and then decreased gradually (Fig. 1). In addition, a splenectomy study indicated that the spleen is responsible for promoting the anti-PEG IgM production elicited by the intravenous injection of PEG-BSA as well as PEGylated liposome (Fig. 3). These results indicate that, like PEGylated liposomes, PEGylated proteins may promote an immune response against PEG. We recently proposed that PEG on the PEGylated liposome stimulates the immune system as one of the TI antigens,^{37,38)} such as bacterial polysaccharide and polyvinyl pyrrolidone.³⁹⁾ Once the PEGylated proteins reach the spleen, they may bind and crosslink to anti-PEG IgM expressed on the surface of B cells, and consequently trigger the production of an anti-PEG IgM. With respect to PEGylated liposome, we showed that lipid dose for injection is one of crucial factors to determine the level of anti-PEG IgM induction; the higher the dose the smaller the anti-PEG IgM induction.⁴⁰⁾ At low amounts of PEGylated protein in spleen the level of crosslinking with B cells might be sufficient to activate the cells specifically; at high amounts of PEGylated protein the cells might rather become anergic. Hence, it is presumed that the physicochemical properties of PEGylated proteins, such as PEG chain length, numbers of the PEG branch and the PEG modification ratio, strongly affect the pharmacokinetics and interactions with B cells and are crucial to determining the degree of anti-PEG IgM production elicited by PEGylated proteins (Fig. 1).

Nakagawa and co-workers reported that, as a result of higher tumor accumulation rates and transgene expression in tumors, the systemic administration of PEG-Ad has a potential for systemic cancer gene therapy that is greater than that of unmodified Ad due to the EPR effect.^{15–18)} In addition, they showed that the PEGylation to Ad can avoid production of specific neutralizing Abs to Ad following intravenous administration.¹⁷⁾ However, in the present study, we observed that a single intravenous injection of PEG-Ad elicits anti-PEG IgM, although neutralizing anti-Ad Abs were not detected (Fig. 5). Such anti-PEG IgM would enhance elimination of PEG-Ad from blood circulation and consequently suppress the therapeutic effect of PEG-Ad by attenuating the level of transgene expression. The relationship between the production of anti-PEG IgM and the therapeutic efficacy of PEG-Ad in therapy after repeated administration remains unclear and should be the subject of future investigation.

CONCLUSION

The results of the present study clearly indicate that anti-PEG IgM can be produced and may limit the efficacy of PEGylated substances such as proteins, Ad vector, and particles in some patients. The immunogenicity of PEGylated substances is usually tested directly against the primary substances, rather than against covalently attached PEG. Our study suggests that the PEG immunogenicity of PEGylated therapeutic agents and particles deserves further investigation.

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