Regular Article

Pegfilgrastim (PEG-G-CSF) Induces Anti-polyethylene Glycol (PEG) IgM via a T Cell-Dependent Mechanism

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Protein-based therapeutics are beginning to be widely used in various clinical settings. Conjugation of polyethylene glycol (PEGylation) to protein therapeutics improves their circulation half-lives in the body. However, we and other groups observed that the initial dose of some PEGylated protein-based therapeutics may induce anti-PEG antibodies (primarily immunoglobulin M (IgM)), resulting in the accelerated clearance of a second dose. The mechanism behind the induction of anti-PEG IgM by PEGylated protein-based therapeutics is still unclear. In this study, we found that Pegfilgrastim (PEG-G-CSF, the PEGylated form of the recombinant human granulocyte colony-stimulating factor) induced anti-PEG IgM in mice when administered via either intravenous or subcutaneous administration. However, the anti-PEG IgM induction was diminished both in athymic nude mice lacking T cells and in splenectomized mice. In addition, anti-PEG IgM production was significantly diminished in the cyclophosphamide-treated mice depleted of B-cells. These results indicate that anti-PEG IgM production by Pegfilgrastim occurs in spleen in a T cell-dependent manner, which differs from anti-PEG IgM induced by PEGylated liposomes. However, B cells, both marginal zone and follicular, are essential for anti-PEG IgM production in both PEGylated preparations.

Key words  PEGylated protein; anti-polyethylene glycol (PEG); immunoglobulin M (IgM); marginal zone (MZ) B cell; accelerated blood clearance (ABC) phenomenon; PEGylated granulocyte colony stimulating factor (PEG-G-CSF)

INTRODUCTION

Protein-based therapeutics have found more and more applications in recent years.¹ Their high activity and selectivity give them advantages in treating disease, however, they also have disadvantages including physicochemical instability, low solubility, proteolytic degradation, relatively short circulating half-life and immunogenicity. PEGylation, the covalent attachment of the hydrophilic polymer polyethylene glycol (PEG), to protein-based therapeutics has been helpful in ameliorating some of these limitations.¹⁻³ In particular, PEGylation prolongs the circulation half-lives of protein therapeutics, and may in some instances increase their biocompatibility and reduce their humoral and cellular immunogenicity. To date, over 20 PEGylated protein-based therapeutics have been approved by the U.S. Food and Drug Administration (FDA) and dozens are in clinical trials.²,³

Nevertheless, PEG, despite of its advantages, also has disadvantages. Some reports have claimed that PEGylated protein-based therapeutics, and liposomal drugs can elicit anti-PEG antibody responses that can adversely affect their circulation half-lives and reduce their therapeutic effects in rodents²,⁴⁻⁵ and in patients.²,⁶,⁷ Furthermore, the potential antigenicity of PEG itself has been observed in healthy individuals who have never received PEGylated therapeutics, but have anti-PEG antibodies, likely derived from PEG-containing food and cosmetics.⁵ There is growing evidence showing that anti-PEG antibodies could be linked to hypersensitivity reactions and non-responsiveness to PEGylated protein-based therapeutics in some clinical cases.² According to the FDA, the PEGylation of biological products to reduce their immunogenicity is not adequate since these products may still be immunogenic.⁸⁻¹³ However, the mechanism(s) underlying anti-PEG antibody induction in PEGylated protein-based therapeutics is still not well understood.

In the last two decades, many studies have helped to explain the mechanism(s) underlying anti-PEG antibody induction and the accelerated blood clearance (ABC) phenomenon to PEGylated nanoparticles such as liposomes.¹⁰⁻¹³ However, the mechanism(s) underlying anti-PEG immunity elicited by PEGylated protein-based therapeutics, are still not well understood, although anti-PEG immunity against PEGylated protein-based therapeutics can reduce the efficacy of PEGylated protein-based therapeutics. In this study, therefore, we investigated some of the mechanism(s) underlying anti-PEG immunity elicited by PEGylated protein-based therapeutics, which differ from anti-PEG IgM induced by PEGylated liposomes. However, B cells, both marginal zone and follicular, are essential for anti-PEG IgM production in both PEGylated preparations.

MATERIALS AND METHODS

Materials  PEG-G-CSF (pegfilgrastim, G-Lasta®) was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Cyclophosphamide (Endoxan®) was purchased from Shionogi & Co., Ltd. (Osaka, Japan). Cholesterol was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan).
Results of splenectomy on anti-PEG IgM production by PEG-G-CSF

Mice were divided into two groups, a splenectomized one and a non-splenectomized one. The former received splenectomy one day before the administration of PEG-G-CSF. The latter received a sham-operation one day before the administration of PEG-G-CSF. The mice were then injected with PEG-G-CSF at a dose of 6 mg/kg either i.v. or s.c. At day 5 after the injection, serum samples were collected. Anti-PEG IgM in the samples was determined with ELISA. Each value represents the mean ± S.D. (n = 3). **p < 0.01 and ***p < 0.001 vs. non-splenectomized mice.

Fig. 1. Effect of Splenectomy on Anti-PEG IgM Production

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Anti-PEG IgM Induction by i.v. Injection of PEG-G-CSF in Nude Mice

BALB/c and BALB/c nu/nu mice received an i.v. injection of PEG-G-CSF at a dose of 6 mg/kg. On Day 5, serum samples were collected. Anti-PEG IgM in the samples was determined with ELISA. Each value represents the mean ± S.D. (n = 4). ***p < 0.001.

Fig. 2. Anti-PEG IgM Induction by i.v. Injection of PEG-G-CSF in Nude Mice
irrespective of the administration route of PEG-G-CSF. These results indicate that the spleen is a major organ inducing the production of anti-PEG IgM, which is consistent with our observations with PEGylated liposomes. 19,20

**Induction of Anti-PEG IgM in Nude Mice** The production of anti-PEG IgM by i.v. administration of PEG-G-CSF was investigated in either immunodeficient athymic BALB/c mice (BALB/c nu/nu) or wild type BALB/c mice (Fig. 2). In the BALB/c nu/nu mice, anti-PEG IgM production was significantly diminished. This observation is consistent with observations for PEG-ovalbumin (OVA),19 but is inconsistent with observations for PEGylated liposomes. 20 This result indicates that the anti-PEG IgM response against PEG-G-CSF is elicited in a T cell-dependent manner.

**Effect of Cyclophosphamide Treatment on Anti-PEG IgM Production** To confirm that cyclophosphamide treatment depletes splenic MZ-B cells, the number of splenic B cells (IgM+ cells) and the numbers of CD21low, CD23+ follicular B (FO-B) cells, MZ-B (CD21high, CD23-) cells were also decreased, but the treatment did not change the number of splenic T-cells (CD3+). I.v. administration of PEG-G-CSF induced anti-PEG IgM production in normal, control BALB/c mice, but barely induced antibodies in the cyclophosphamide-treated mice (Fig. 3B). These results indicate that splenic B cells are responsible for production of anti-PEG IgM against administered PEG-G-CSF.

**DISCUSSION**

PEGylated protein-based therapeutics is widely applied for the treatment of a range of indications and new ones continue to be developed. Therefore, the observation of anti-PEG immunity and its negative consequences on the therapeutic efficacy/safety of PEGylated protein-based therapeutics could impact their clinical use. PEG-related immunological responses are received increasing attention. 5,21,22 Although the mechanism(s) underlying anti-PEG IgM induction have been reported for PEGylated nanoparticles such as liposomes, 12,23,24 data regarding the mechanism underlying the immunogenicity of PEG-conjugated protein-based therapeutics is still scarce. In the current study, we showed in control mice that PEG-G-CSF (pegfilgrastim, G-Lasta®) induced the production of anti-PEG IgM in spleen in a T cell-dependent manner (Fig. 2) and that the spleen was the only source of the anti-PEG IgM production (Fig. 1), and that B cells (FO-B cells and MZ-B cells) were essential for the antibody production (Fig. 3). To the best of our knowledge, this is the first report to suggest the involvement of the splenic B cells (FO-B cells and MZ-B cells) and T cells in the induction of anti-PEG IgM by an FDA-approved PEGylated protein-based therapeutic, Pegfilgrastim (PEG-G-CSF).

In the present study, we demonstrated that i.v. injection of PEG-G-CSF induced stronger anti-PEG IgM response than the s.c. one (Fig. 1). It is known that route of administration affects distribution kinetics of injected particles. After s.c. administration, only a small fraction of the administered dose of PEG-G-CSF reaches spleen, due to slow, incomplete absorption from the site of injection. By contrary, following i.v. administration, the whole dose would be available into the blood circulation and a large fraction of PEG-G-CSF would rapidly be distributed to spleen. The preferential accumulation of PEG-G-CSF in the spleen following i.v. administration, compared to that following s.c. administration, can explain the difference in anti-PEG IgM response.

The T cell-dependent mechanism observed in this study was similar to that for PEG-OVA,19 but was not the same as that for PEGylated liposomes, which induces anti-PEG IgM production in a T cell-independent manner. 20 In our earlier studies, we hypothesized the mechanism behind anti-PEG IgM induction by PEGylated liposomes is as follows: once an initial dose of PEGylated liposomes reaches the spleen, the liposomes bind and crosslink to surface IgM on reactive MZ-B
cells in the spleen and consequently trigger the production of an anti-PEG IgM that is independent of T cell help.\(^3,23,24\) It is known that infectious pathogens are complex and composed of both T cell-dependent and T cell-independent epitopes. PEG in the PEGylated liposomes is a T cell-independent antigen,\(^20\) while the protein in PEGylated protein-based therapeutics is a T cell-dependent antigen\(^19\) (Fig. 1). MZ-B cells are likely to be heterogeneous and multi-reactive, reacting with a wide variety of pathogen-associated antigens. In addition to their ability to mount a local antibody response against T cell-independent antigens, MZ-B cells can participate in T cell-dependent immune responses through the capture and import of blood-borne antigens to follicular areas of the spleen.\(^25\) In the present study, treatment with cyclophosphamide decreased the number of splenic MZ-B cells (Fig. 3A) as previously reported\(^26–28\) and reduced anti-PEG IgM production by PEG-G-CSF (Fig. 3B). Therefore, MZ-B cells in the spleen B cells may play a crucial role in the anti-PEG IgM production against, not only PEGylated liposomes, but also PEGylated protein-based therapeutics.

T lymphocyte is composed of different cell subsets; T-helper (Th), Th1, Th2, Th17 and Treg lymphocytes. These T lymphocyte subsets form a complex immune network that participates to both cellular and humoral immune responses via secreting a variety of cytokines. Among them, Th2 lymphocytes are known to be responsible for humoral immunity against extracellular pathogens by cooperation with B lymphocytes secreting antibodies through the release of cytokines, such as interleukin 4 (IL-4), which support B-cell development and production of antibodies. In the current study, Th2 cells are most likely to be the contributing cells to anti-PEG IgM production. However, the exact contribution of Th2 cells to the anti-PEG IgM production against PEGylated protein-based therapeutics is still uncertain. The investigation is still continued in our laboratory.

Since cyclophosphamide treatment also diminished the number of FO-B cell in spleen (Fig. 3A), the contribution of FO-B cells to anti-PEG IgM production cannot be negligible. Splenic FO-B cells are known to differentiate into long-lived plasma cells that are capable of secreting the IgG isotype of antibodies for several months in the absence of any cell division or re-exposure to antigen.\(^29,30\) The MZ-B cells have been shown to be the major producers of IgM antibodies.\(^31,32\) The anti-PEG antibodies induced by PEG-G-CSF were mainly of the IgM type (Fig. 1) and no anti-PEG IgG antibodies was produced by PEG-G-CSF up to day 14 (data not shown). This suggests that FO-B cells have little or no contribution, relative to MZ-B cells, to the production of anti-PEG IgM against PEG-G-CSF.

The logic of the hapten-carrier system would account for anti-PEG antibody production against different PEGylated products.\(^33,34\) In this study, high dose of PEG-G-CSF could trigger anti-PEG IgM production in normal BALB/c mice (Figs. 1, 2, 3) without eliciting any anti-G-CSF IgM (data not shown). These results support the concept that the production of antibodies occurs only against PEG on the conjugates where PEG acts as a hapten. A hapten is defined a small molecule that cannot elicit an immune response unless it is covalently attached to a larger molecule (proteins, peptides, or nanoparticles), which then makes it reactive to the immune system.\(^35\) This haptenic characteristic of PEG is likely dependent on the molecular weight and the nature/immunogenicity of the carrier and/or the presence of adjuvants.\(^36–38\) Previous reports have shown that proteins conjugated with a hapten perform as TD antigens,\(^39\) while non protein carriers, such as liposome and polysaccharide, conjugated with a hapten act as TI antigens.\(^40\) This might explain the controversy surrounding the mechanism of antibody production against PEG-G-CSF and PEGylated liposome in various developmental and clinical situations.

In animal studies, the incidence of anti-PEG immunogenicity and the ABC phenomenon against PEGylated therapeutics has been found to be varied depending on animal species used. Such variation could be attributed, at least in part, to the difference in sensitivities of animals to the immune responses as well as the differences in pharmacoynamics of drugs. Splenic MZ-B cells have been reported to play a crucial role in the production of anti-PEG antibodies by PEGylated liposomes in rodents and pigs.\(^33,24\) However, there could be a difference in the mechanism of anti-PEG antibodies production between human and animals, especially that the anti-PEG antibody production in human is more skewed toward the IgG isotype.\(^41\) Therefore, difference between animal species should be considered an important issue that must be addressed by pre-clinical study of the PEGylated formulation.

**CONCLUSION**

Pegfilgrastim (PEG-G-CSF) induces anti-PEG IgM production with the help of T cells and B cells (FO-B cells and MZ-B cells) in the spleen. Our study may provide a step forward towards uncovering the mechanism underlying anti-PEG IgM production by PEGylated protein-based therapeutics.

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**Conflict of Interest** The authors declare no conflict of interest.

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