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EXPERT REVIEW

The Immunogenicity of Polyethylene Glycol: Facts and Fiction

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ABSTRACT An increasing number of pegylated therapeutic proteins and drug targeting compounds are being introduced in the clinic. Pegylation is intended to increase circulation time and to reduce an immunogenic response. Recently however a number of publications have appeared claiming that the polyethylene glycol (PEG) moiety of these products in itself may be immunogenic and that the induced anti-PEG antibodies are linked to enhanced blood clearance and reduced efficacy of the products. A critical review of the literature shows that most, if not all assays for anti-PEG antibodies are flawed and lack specificity. Also the biological effects induced by anti-PEG antibodies lack the characteristics of a *bona fide* antibody reaction. Standardization of the anti-PEG assays and the development of reference sera are urgently needed.

KEY WORDS immune assays · immunogenicity · pegylation · therapeutic proteins

ABBREVIATIONS

ABC	Accelerated blood clearance
BSA	Bovine serum albumin
ELISA	Enzyme linked immunosorbent assay
EMA	European medicines agency
FDA	Food and drug administration
Ig	Immunoglobulin
OVA	Ovalbumin
pDNA	Plasmid DNA
PEG	Polyethylene glycol

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INTRODUCTION

Pegylation is frequently used to improve the clinical properties of therapeutic proteins (1,2). It increases the protein size, inhibits proteolysis and renal filtration and thereby improves the pharmacokinetics of the protein drug. In addition, polyethylene glycol (PEG) has a simple structure and is chemically inert. It also has an excellent solubility and no effect on protein conformation. Pegylation is therefore considered an effective method to improve protein stability and to decrease the immunogenic potential of the modified proteins.

Pegylation is also used to improve drug delivery systems (3). The long circulating property of PEG coated liposomes as well as other colloidal nanoparticles has been generally attributed to suppression of protein adsorption onto their surfaces which renders them invisible (or Stealth) for macrophages. In addition PEG has very low toxicity and because of its simple structure is assumed to be of low immunogenicity itself.

Several pegylated products have been approved by the FDA, EMA and other regulatory authorities and are use clinically with success. These products include PegIntron®, a pegylated form of interferon-alpha 2b, Pegasys®, a pegylated form of interferon- alpha 2a, Neulasta®, a pegylated form of granulocyte colony stimulating factor and Mircera®, a pegylated epoetin-beta. Long-term treatment of hundreds of thousands of patients with these products has confirmed their safety.

This very positive clinical experience with marketed pegylated therapeutic proteins is in sharp contrast with an increasing number of reports claiming that PEG can be highly immunogenic, and might compromise efficacy and safety of protein drugs (4). Moreover there are reports suggesting an increasing incidence of anti-PEG antibodies in normal donors. It has been suggested that these anti-PEG

antibodies prime an immune response if these individuals are treated with a pegylated product (5), and might therefore have a negative clinical effect. Besides reports claiming that immunogenicity of PEG might affect safety of protein drugs, other studies have implied that the phenomenon of accelerated blood clearance (ABC) seen in animal models treated with pegylated liposomal products is linked to the induction of anti-PEG antibodies (6).

Potential immunogenicity of PEG has therefore many implications for both the development of pegylated therapeutic proteins and drug delivery systems. It may compromise the use of animal studies to evaluate the safety and pharmacokinetic/pharmacodynamic properties of these products. In addition, immunogenicity may reduce safety and efficacy in patients. It also has regulatory consequences because the FDA and other regulatory agencies expect the immunogenicity of all components of hybrid molecules, such as PEG, to be evaluated separately. However designing and validating assays to test for antibodies against these individual components, and PEG in particular is a major challenge.

In this paper we will critically review the current data on the incidence of anti-PEG antibodies in the general population and on the immunogenicity of PEG in both animals and patients. We will start with reviewing the assays used for measuring antibodies to PEG.

ASSAYS FOR ANTIBODIES TO PEG

It is difficult to reconstruct the performance of the assays used for the original observations of anti-PEG antibodies in normal donors or patients and animals treated with pegylated products some 30 years ago. Methods used then, such as gel diffusion and haemagglutination, are now mostly obsolete (7,8). Also, in all recent papers on the occurrence of anti-PEG antibodies the details on the methods used, their specifications and validation are lacking and cannot be traced back in the papers referenced as source of the original method.

The enzyme linked immunosorbent assay (ELISA) is currently the most widely used format to detect antibodies to PEG. As example of the poor description of these assay in papers claiming immunogenicity of PEG, we have selected the recent paper by Shimizu and colleagues reporting about the immunogenicity of pegylated bovine serum albumin (BSA) and ovalbumin (OVA) in rats (9). The method they use is straightforward. Microtiter plates are coated with 1 μg of pegylated BSA and after washing, blocking with BSA, and washing and drying, diluted sera are added to the wells. Wells are again washed after incubation with sera and antibodies are captured with pegylated BSA. Labelled goat anti human IgG or IgM is then used to detect the different antibody isotypes.

For this assay there are no data given on essential characteristics such as limit of detection, limit of quantification, sensitivity, specificity, reproducibility, accuracy and precision. There is no mention of the sources and compositions of the positive and negative controls, of the standard and of the characteristics of the standard curve. In the paper of Shimizu the binding activity of the antisera is sometimes expressed as % of the positive control and sometimes reported as extinction value. In addition, extinction results obtained with different assays using different products as coating antigens are directly compared without any data on how these results were normalized in order to allow such direct comparisons.

There are also conflicting reports concerning the applicability of different steps and reagents used in the ELISA. Shimizu and colleagues use Tween 20 in the various washing and incubation buffers. Tween 20 is added in washing buffers of these assays to prevent aspecific binding, however other papers reported that this detergent interferes with antibody binding because of an assumed cross-reactivity of the anti-PEG antibodies with Tween 20 (10,11). Shimizu and colleagues also used direct coating of their pegylated products to the plates while others have reported problems with this procedure (9). One group showed a lack of binding of unconjugated or non-activated PEG to the well and another group found the dynamic range of the assay insufficient in case the PEG was directly coated to plate (12,16)

The best documented anti-PEG antibody assay is the commercially available ELISA type bridging assay recently described by Liu and colleagues (12). Nevertheless, also this paper lacks essential information such as the characteristics of the ANPEG 1 mouse IgM monoclonal antibody used as quality control and as standard. In addition a full dose response curve is lacking and PEG moieties of different length and chemical functionalization are used to show specificity, although such an approach is not suitable for human polyclonal antisera.

Indeed, researchers from Covance recently published a comparison of different ELISA formats to detect anti-PEG antibodies as reported in the literature, including the commercially available ELISA, and found these to have a high level of non-specific binding (13). Also they were unable to reproducibly confirm binding in alleged positive human, monkey or rabbit monoclonal antibody diluted in human serum. These results show that standardization of the assay methodology for antibodies to PEG is needed and also show that well characterized reference sera and standards should be developed and made generally available.

ANTI-PEG ANTIBODIES IN PATIENTS AND BLOOD DONORS

In 1984, Richter and Akerblom reported anti-PEG antibodies in 0.2% of healthy blood donors and in 3.3% of

untreated allergic patients (8). During hyposensitization with methoxy-PEG-modified ragweed extract and honey bee venom, respectively, anti-PEG IgM antibodies were detected in 50% of the patients directly after the first treatment course. After 2 years of treatment the percentage of antibody positive patients declined to 28.5%. The assay used to detect anti-PEG antibodies was passive hemagglutination, where PEG-coated erythrocytes were incubated with serial dilutions of human sera. After incubating for 2 h settling was measured and recorded as the reciprocal of the highest dilution giving complete hemagglutination. A titer of 32 or more was considered positive for anti-PEG antibodies.

Garratty and colleagues reported a high occurrence (22%–25%) of anti-PEG IgM antibodies in normal donors (14,15). These IgM antibodies were also identified with a haemagglutination assay using PEG-coated erythrocytes. This higher incidence of anti-PEG antibodies in the normal population compared to the incidence found by Richter and Akerblom is often cited in the literature as evidence of an increasing incidence of pre-existing anti-PEG antibodies which may compromise the use of pegylated biopharmaceuticals and other pegylated forms of therapy. However, although comparable assay technologies were used, the lack of reference sera and a lack of data on the validation and specifications of the assay make it impossible to directly compare results and draw the conclusion of an increasing incidence of anti-PEG antibodies in the healthy donor population. The data by Garratty are also in sharp contrast with the recent report by Liu and colleagues in which about 4% of 350 healthy blood donors were found antibody positive using the commercially available ELISA assay described above (12).

The group of Garratty (5) also reported that in patients with acute lymphoblastic leukemia treated with pegylated asparginase, the lack of enzyme activity in the blood was associated with the presence of anti-PEG antibodies.

Ganson and colleagues investigated the immunogenicity of subcutaneous injections of pegylated uricase in 13 patients with gout. In five subjects, plasma uricase activity could not be detected beyond 10 days after injection. This uricase clearance was associated with the appearance of low titers of anti-PEG IgM and IgG (16). They used an ELISA format to detect anti-PEG, in which PEG-uricase was used to coat the plates. Also here Tween containing buffer was used for some of the washing steps, and as positive control a sample from a study subject with the highest ELISA response in the initial screen was used. They checked for specificity of the antibodies by spiking with pegylated uricase.

Tillmann and colleagues found a high frequency of anti-PEG antibodies in patients with hepatitis C. However, these antibodies had no effect on the efficacy of PEG-interferon used to treat this form of chronic hepatitis (17). They used the same assay format as described above for the Ganson study.

ANTI-PEG ANTIBODIES IN EXPERIMENTAL ANIMALS

In the first paper reporting on the induction of antibodies to PEG in animals, rabbits were challenged with different pegylated products (7). Only monomethoxy-PEG-ovalbumin administered with complete Freund's adjuvant induced anti-PEG antibodies, whereas PEG given alone was not immunogenic. The antibodies were detected with gel diffusion and passive haemagglutination assays. Although the paper did not discuss the specificity and sensitivity of the assays, these techniques are now obsolete and less sensitive than the currently used immune assays formats. It might therefore be of use to repeat these experiments using more up to date assay methodology.

Sroda and colleagues (2005) also used rabbits but they challenged the animals with pegylated liposomes (spherical particles composed of a lipid bilayer) (18). Blotting assays and in-house fluorescent dye release methods were used to detect anti-PEG antibodies. Although a serum factor on the surface of the liposomes reacted with an anti-human IgG antibody, its molecular weight was 55 kD, which is too small for an IgG molecule. Moreover, the binding of the antiserum was reduced by heating at 56°C, which is routinely used to destroy complement factors. This suggests that a serum component related to the complement system was detected, and further studies should be done to conclude formation of anti-PEG antibodies.

In a more recent paper by Tagami and co-workers, PEG coated liposomes or PEG coated pDNA lipoplexes (deoxyribonucleic acid-liposome complexes) were injected in conventional and nude mice (19). The immune response against PEG was monitored with a classical direct ELISA. The liposomes induced an IgM response that peaked 5 days after challenge, and this response was reduced by increasing the amount of liposomes injected. Also PEG coated pDNA-lipoplexes induced the production of IgM, although the level did not decrease with increased dosing. A reduced response was seen if the lipoplexes carried GpC free DNA, which is considered to prevent the induction of an innate immune response. In nude mice lacking T-cells, an enhanced anti-PEG IgM response was seen.

Kaminskas and colleagues (2011) challenged rats intravenously with pegylated liposomes or pegylated micelles (spherical particles composed of polymeric molecules) (20). A classical IgM ELISA was used, but without the addition of Tween 20 in the washing buffer since this was reported to abolish the signals in the assay. Both pegylated products induced an anti-PEG IgM antibody response peaking at day 5. Increasing the size of the liposomes reduced the IgM response. Although the micelles induced an immune response and an enhanced clearance was seen with a second injection of micelles, liposomes were cleared more rapidly in mice pre-treated with micelles.

Sherman and colleagues (2012) used different proteins conjugated to PEG with a variation of terminal chemical groups (11). They immunized rabbits with these products in complete Freund's adjuvant in the first immunization and in incomplete Freund's adjuvant in the follow-up immunisations. A classical ELISA was used to detect antibodies and displacement of the binding signal with different products was used to show specificity of the reaction. They also reported a loss of reactivity of the antisera when Tween 20 was used in the washing and dilution buffer.

Zhang and colleagues (2012) immunized rats with different forms of pegylated canine uricase (21). Only the pegylated aggregated form induced anti-PEG IgM antibodies with a peak at day 7. No immune reaction was seen after subsequent injections. The assay was a classical ELISA and the antibodies did not react with non-pegylated uricase but were binding pegylated albumin, indicating specificity against PEG.

Shimizu (2012) induced an IgM response with 1 μg of pegylated BSA or OVA injected intravenously in rats (9). They also induced a response with a pegylated adenovirus vector. The assay details are described in more detail earlier in this review. The peak of the antibody response was observed on day 5. However, no reaction was seen to unpegylated OVA or BSA. The antibodies induced by the pegylated products were reported to react with pegylated liposomes.

The animal data show conflicting results concerning the immunogenicity of PEG. In all cases a pegylated product was necessary to induce an antibody response, while PEG alone was not immunogenic. The immunogenic properties were dependent on the protein as well as the size and the chemical composition of the terminal part of the PEG moiety. Sometimes a strong adjuvant like complete Freund's was necessary to induce a response, while in other cases 1 μg intravenously administered pegylated protein alone was sufficient to induce a response, while vaccines need a 50–100 times higher dose, the use of an adjuvant and repeated subcutaneous administrations. Also the kinetics of the response with a peak at 5–7 days, a lack of response to subsequent administrations, the inverse dose–response, the increased response in nude mice and the lack of identification of the induced binding factor as an immune globulin are all contradicting a bona fide antibody response.

PEGYLATED DRUG DELIVERY SYSTEMS

Accelerated Blood Clearance (ABC)

PEG induced antibodies are also implicated in the increased clearance of pegylated liposomes after repeated administration observed in animal studies. However this phenomenon is not without controversy. In a study in which rabbits received serial injections of pegylated liposomes 6 weeks apart no differences in pharmacokinetics were reported (22). Oussoren reported unchanged pharmacokinetics in rats treated with four doses of pegylated liposomes at 24 or 48 h intervals (23).

In other studies large pharmacokinetic changes with repeated injections of pegylated liposomes were reported (24). Also prior dosing of "empty" pegylated liposomes was reported to substantially decrease the pharmacokinetics of a second dose of drug loaded liposomes in rats and rhesus monkeys (25). This enhanced blood clearance reached a maximum effect at 4–10 days after the first dose in rats and mice.

Several lines of evidence were presented to explain the ABC as antibody mediated. In rats, ABC of pegylated liposomes was seen after transfusion with serum obtained from animals that received pegylated liposomes 1 week earlier, indicating that soluble serum factors were involved. The serum factor was identified of being a heat labile protein with a kD of 150 (26).

In a number of papers it was argued that "empty" pegylated liposomes could give rise to an abundance of anti-PEG IgM (6,27). The mechanism proposed for the induction of the ABC phenomenon involved anti-PEG IgM production in the spleen which could selectively bind to PEG after a second dose of these liposomes was given. As a result the complement system would be activated, leading to opsonisation of the second dose PEG liposomes by the Kupffer cells in liver. The PEG molecule has been suggested to induce IgM in a T-cell independent mechanism by directly activating marginal zone B-cells, in a similar fashion as has been reported for highly repetitive structures (28).

Besides doubts about the specificity of the anti-PEG IgM assay that was employed, a number of observations makes the antibody mediated enhanced clearance rather unlikely. There was an inverse relationship with dose and density of the PEG molecules on the liposomes, which contradicts the observations with other repetitive structures capable of directly activating B-cells. Also the kinetics of the ABC does not reflect the kinetics of an IgM response. The peak of the enhanced clearance is a few days after the first injection and seems to wane after a few weeks. Also a third injection hardly showed an effect on clearance.

An even more remarkable observation on the kinetics of the antibody response was reported by Judge and colleagues (2005) (29). In contrast with the observations of the Japanese group they observed an IgG response peaking at day 20 after treating mice with pegylated liposomes loaded with DNA. This was preceded by an IgM response with an apparent peak at day 5, which disappeared at day 14. Their IgG/IgM assay was a classical ELISA with direct binding of PEG to the wells of the plates.

The study of Kaminskas and colleagues (2011) argued that pegylated micelles apparently induced IgM although at a lower level than pegylated liposomes (20). However the micelles did not show an accelerated clearance if injected 7 days after treatment with either pegylated liposomes or micelles. It is difficult to understand why an IgM antibody capable of clearing pegylated liposomes would be unable to clear a pegylated micelle.

Two mechanisms independent of a specific antibody response to PEG may explain the alleged immune response induced by pegylation. PEG is reported to be able to activate complement. Hamad and colleagues reported that highly concentrated monodisperse endotoxin-free PEGs can induce complement activation and increase complement factors in human serum within minutes (30). The same has been reported for pegylated liposomes (Van den Hoven J. Thesis. Utrecht; 2012). In addition pegylated surfaces have been reported to aspecifically bind IgM and IgG that, together with the complement factors, may lead to the formation of a complex including pegylated product, antibodies and other serum factors (14). Subsequent uptake by macrophages of these complexes may explain the increased blood clearance.

The nonspecific binding of IgG and IgM by pegylated products also makes the validation of the specificity of any anti-PEG binding assay extremely difficult. And as long as well validated assays for anti-PEG antibodies are not available, any conclusion regarding the immunogenicity of PEG is premature.

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