EXPERT OPINION

Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents

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In contrast to the accepted general assumption that polyethylene glycol (PEG) is non-immunogenic and non-antigenic, animal studies clearly showed that uricase, ovalbumin and some other PEGylated agents can elicit antibody formation against PEG (anti-PEG). In humans, anti-PEG may limit therapeutic efficacy and/or reduce tolerance of PEG-asparaginase (PEG-ASNase) in patients with acute lymphoblastic leukemia and of pegloticase in patients with chronic gout, but did not impair hyposensitization of allergic patients with mPEG-modified ragweed extract or honeybee venom or the response to PEG-IFN in patients with hepatitis C. Of major importance is the recent finding of a 22 – 25% occurrence of anti-PEG in healthy blood donors, compared with a very low 0.2% occurrence two decades earlier. This increase may be due to an improvement of the limit of detection of antibodies during the years and to greater exposure to PEG and PEG-containing compounds in cosmetics, pharmaceuticals and processed food products. These results raise obvious concerns regarding the efficacy of PEG-conjugated drugs for a subset of patients. To address these concerns, the immunogenicity and antigenicity of approved PEGylated compounds should be carefully examined in humans. With all these data in hand, patients should be pre-screened and monitored for anti-PEG prior to and throughout a course of treatment with a PEGylated compound. Finally, protein conjugates with the poorly immunogenic hydroxy-PEG sequence or other hydrophilic polymers are in early phases of development and may represent an alternative to immunogenic PEGylated proteins.

Keywords: antigenicity, anti-PEG, immunogenicity, PEGylated proteins, PEGylation, polyethylene glycol

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1. Introduction

PEGylation technology, the covalent attachment of polyethylene glycol (PEG) to active recombinant proteins and peptides, allows significant extension of their biological half-life, reduction in toxicity, antigenicity and immunogenicity while sustaining therapeutic effectiveness [1]. PEG has been covalently attached to oligonucleotides and small organic molecules [2], as well as to phospholipids for PEGylated liposomes [3]. PEGylated modifications of recombinant proteins and antibodies became the golden standard of the technology due to increased solubility, stability, masking humoral and cellular immunogenicity, protease protection, controlled release, reduced absorption, reduced injection volume and ease of conjugational linking as reviewed by Chen *et al.* [4]. Since the approval of PEG-adenosine deaminase for severe combined immunodeficiency syndrome in 1990, at least 12 therapeutic

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Table 1. PEGylated products in clinical practice.

Conjugate	Indication
PEG-adenosine deaminase (Adagen®)	SCID
PEG-asparaginase (Oncaspar®)	Leukemia
*PEG-IFN-α2b (PegIntron®)	Hepatitis C
*PEG-IFN-α2a (Pegasys®)	Hepatitis C
PEG-human growth hormone receptor antagonist (Somavert®)	Acromegaly
*PEG-G-CSF (Neulasta®)	Neutropenia
PEG-anti-VEGF aptamer (Pegaptanib, Macugen™)	Wet age-related macular degeneration
PEG-erythropoietin analogs (Mircera®, Omontys®)	Anemia associated with chronic kidney disease
PEG-anti-TNF Fab′ (Cimzia®)	Rheumatoid arthritis and Crohn's disease
PEG-uricase (Pegloticase; Krystexxa®)	Chronic gout
PEG-liposome/doxorubicin (Doxil®/Caelyx®)	cancer

For details, see Pasut and Veronese [36].

*Blockbuster, as revealed by the market analysis.

PEG: Polyethylene glycol; SCID: Severe combined immunodeficiency disease.

PEGylated agents, including 3 blockbusters have been approved (**Table 1**), with several more in development. PEGylation technology is considered to be one of the most advanced treatment approaches [4]. It is therefore disappointing that pre-existing and induced antibodies against PEG (anti-PEG) have been reported, suggesting that PEG is both immunogenic and antigenic [5,6].

2. Anti-PEG in animal models

In animal models, PEG is generally considered to be non-immunogenic by itself, which may in part be due to rapid renal clearance of the unconjugated polymer. A strong anti-PEG immune response was found with some PEGylated proteins, particularly ovalbumin [7] and uricase [8]. The haptogenic character of PEG depended on its molecular weight, the immunogenicity of the anchoring protein and the presence of adjuvants [6]. Although different epitopes were identified in the PEG moiety (see Section 7), PEG itself is considered non-immunogenic, thus suggesting a complex involvement of the conjugated protein/liposomes.

Several authors reported that PEGylated liposomes elicit a strong immune response that results in the rapid blood clearance of subsequent PEG-liposome doses in mice, rats, rabbits and monkeys [9-12]. This phenomenon, known as accelerated blood clearance (ABC) is due to an anti-PEG immune response [9,11,12]. Surprisingly, robust, long-lived anti-PEG response was found with PEG-liposomes encapsulating nucleic acid [11]. The magnitude of the response was sufficient to induce significant morbidity and, in some instances, mortality [10].

In contrast to the above studies, development of antibodies against PEG-methioninase did not result in any immunological reaction or decreased activity in monkeys [13], although no attempt was done to discriminate between PEG and methioninase antibodies.

3. Anti-PEG in humans

In humans, anti-PEG did not impair hyposensitization treatment of allergic patients with PEG-modified allergens [14] or the response to PEG-IFN in patients with hepatitis C [15]. It is perhaps not surprising that anti-PEG did not affect hyposensitization therapy to ragweed pollen or honeybee venom as the treatment comprised increased doses of an allergen with an aim of inducing immunologic tolerance [14]. However, the presence of anti-PEG was shown to correlate closely to rapid clearance of PEG-asparaginase in patients with acute lymphoblastic leukemia and may limit therapeutic efficacy [16]. Moreover, anti-Peg was detected in 89% of patients with refractory gout and treated with Pegloticase, whose response to treatment was inversely correlated to the anti-PEG titers [17]. These data are not surprising as anti-PEG induction may depend on the nature of the protein, with uricase being strongly haptogenic. Hamad *et al.* [18] reported that highly concentrated monodisperse endotoxin-free PEGs can generate complement activation products in human serum on a time scale of minutes.

A high prevalence of anti-PEG (44%) was found in patients with hepatitis C [15]. In healthy blood donors with no known prior exposure to PEG, prevalence of anti-PEG has been reported up to 25% [5], which contrasts with a 0.2% occurrence reported over 20 years ago by Richter and Akerblom [14]. This increase may be due to an improvement of the limit of detection of antibodies during the years and to greater exposure to PEG in cosmetics, pharmaceuticals and processed foods [6], and requires confirmation by further studies.

4. Precautions to take concerning PEG-conjugated drugs

The above results raise concerns regarding the efficacy of PEG-conjugated drugs for a subset of patients. It is still too early to provide guidelines concerning the precautions to take with PEGylated compounds in clinical practice. To address these concerns, the cross-reactivity between anti-PEG and different PEGylated proteins should be investigated *in vitro* and evaluated in animal models. The immunogenicity of approved PEGylated compounds (particularly those positive in *in vitro* and animal tests) should be carefully examined in humans. With all these data in hand, some patients should perhaps be pre-screened and monitored for anti-PEG prior to and throughout a course of treatment with a PEGylated compound. Moreover, PEG is non-biodegradable [4,19] and the impact of the persistence of the PEG moiety should also be monitored. Non-immunogenic alternatives to PEG should be seriously addressed for recombinant proteins and therapeutics in general.

5. The case of uricase

Treatment of gout can be challenging in patients intolerant or refractory to available urate-lowering therapy, mainly xanthine oxidase inhibitors. In this context, uricase, which converts urate to allantoin, represents an interesting option. Unfortunately, the native enzyme is highly immunogenic [20]. PEGylation nullified the immune response of uricase making it safer [21]. In 2010, the US Food and Drug Administration (FDA) approved pegloticase, a PEGylated uric acid-specific enzyme, for treating chronic gout in adults with disease refractory to conventional therapy. Pegloticase is probably the most powerful drug to debulk the urate load in patients with severe gout, but its use is hampered by occurrence of PEG antibodies, resulting in increased drug clearance, loss of efficacy and increased risk of subsequent infusion reactions [17]. Given the burden of refractory gout, researchers have investigated the immunogenicity of other PEG-uricases in development, such as pegsiticase [22] and have explored the means to reduce uricase immunogenicity. da Silva Freitas *et al.* [23] have developed recombinant uricase from *Candida* sp. PEGylated with mPEG-*p*-nitrophenol carbonate or mPEG-cyanuric chloride. When injected repeatedly in mice for 21 days, the uricase did not induce detectable antibody response. However, the use of reactive PEG derivatives with a leaving group after binding (e.g., succinimidyl derivatives) are preferable as functional moieties that remain after binding, such as cyanuric chloride, have potential immunogenicity and can remain reactive toward thiol groups after conjugation [11]. A different approach was used by Tan *et al.* [24] by encapsulating an uricase from *Candida* in lipid vesicles.

Finally, another possibility to generate a less immunogenic uricase could be to develop a 'reactivated' human uricase. Indeed, due to a nonsense codon inserted into the uricase gene, this enzyme is produced as a truncated, 10 amino acids long, inactive fragment in humans and apes [24,25]. Therefore, 'reactivation' of the human uricase, by eliminating nonsense mutations, could be another interesting option.

It is therefore expected that future research developments will reduce the immunogenicity of uricase. This is an issue which has been addressed successfully for many therapeutic proteins [26]. It consists of identifying and removing antigenic units (epitopes) that can be recognized by antibodies, B cells or T cells. Highly sophisticated recombinant DNA technology, such as site-directed mutagenesis [27], allows amino acid substitutions in a particular epitope to make the encoded protein less immunogenic without altering its activity.

6. The case of PEG-coated red blood cells to produce universal/stealth donor red cells suitable for transfusion

In the late 1990s, several groups became interested in binding PEG to the red blood cell (RBC) membrane to cover red cell antigens. It was suggested that such RBCs might be used as universal/stealth RBCs, suitable for transfusion [28]. In 1997, one of the authors (JKA) reported that PEG coating of RBCs dramatically reduced aggregation and low shear viscosity of RBCs suspended in plasma [29]. Armstrong *et al.* [29] suggested that this might be of significant benefit in treating diseases characterized by vaso-occlusion or impaired blood flow (e.g., myocardial infarction and sickle cell disease). They also noted that Rh(D)+ PEG-coated RBCs did not react with anti-Rh(D) and that ABO reactivity was diminished considerably.

During the next 10 years, Garratty [28] and other investigators studied the possibility of blocking all RBC antigens by PEGylation of RBCs using both linear PEG and multi-armed reactive PEGs cross-linked with multi-armed PEG-amines. However, *in vivo* studies in rabbits and *in vitro* testing of human sera were discouraging because of antigenicity/immunogenicity of PEG-RBCs, negating the benefits of masking RBC blood group antigens with PEG [5,28].

7. Hydroxy-PEG, a safer alternative to methoxy-PEGylation?

All currently approved PEGylated therapeutic products contain methoxy-PEG (mPEG) [30,31]. Using a rabbit model, Sherman *et al.* [30] showed reduced immunogenicity of hydroxy-PEG (HO-PEG) derivatives compared with mPEG derivatives of porcine uricase (and other proteins). The results were consistent with the hypothesis that antibodies with high affinity for methoxy groups contribute to the loss of efficacy of mPEG conjugates. However, previous studies have shown that both pre-existing and induced anti-PEG binds also to the PEG backbone [5,7]. Further studies are therefore required to determine how HO-PEG derivatives compare with mPEG in humans.

8. Alternative methods to PEGylation

Alternative methods to PEGylation are now emerging, based on the idea to substitute PEG by hydrophilic and flexible polypeptide chains (see review by Chen *et al.* [4]). XTEN technology (half-life extension technology) is based on polypeptide chains of varying length composed of alanine, glutamic acid, glycine, proline, serine and threonine [32]. XTEN sequences have been shown to be safe and poorly immunogenic in a number of animal species [32]. An exenatide–XTEN fusion construct (VRS-859) for diabetes type II and a growth hormone–XTEN fusion construct (VRS 317) for growth hormone deficiency are undergoing Phase I trials [33].

PASylation® technology is based on non-immunogenic polypeptide sequences comprising the natural amino acids proline, serine and alanine (PAS) [34]. Several PASylated compounds, including growth hormone, leptin and IFN-α are under preclinical development [35].

PolyXen® technology uses the biopolymer polysialic acid [4]. It has two disclosed drug candidates, an erythropoietin–PAS fusion construct (ErepoXen) presently in Phase II of clinical development and SuliXen, a PSA–insulin presently in Phase I trial.

9. Conclusions

In conclusion, in contrast to the popular assumption that PEG is biologically inert, PEG is both immunogenic and antigenic. While PEGylation of therapeutic agents have shown, and will continue to show, great value in medicine to address toxicity, immunogenicity and rapid clearance of an unconjugated drug while maintaining efficacy in the treatment of many diseases, it is possible that a subset of patients with anti-PEG may not benefit from treatment with PEG-conjugated agents. Further research is warranted to evaluate the therapeutic risk and determine the impact of anti-PEG, and when anti-PEG is detected, to develop an effective course and method of treatment or to consider replacement with an effective non-PEGylated drug. Finally, safer and poorly immunogenic alternatives to mPEG for therapeutics delivery (OH-PEG, XTEN or PAS polypeptides) are actually under early phases of development.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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