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Potential induction of anti-PEG antibodies and complement activation toward PEGylated therapeutics

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Conjugation of polyethylene glycol (PEG) to therapeutics has proven to be an effective approach to increase the serum half-life. However, the increased use of PEGylated therapeutics has resulted in unexpected immune-mediated side-effects. There are claims that these are caused by anti-PEG antibodies inducing rapid clearance. These claims are however hampered by the lack of standardized and well-validated antibody assays. PEGylation has also been associated with the activation of the complement system causing severe hypersensitivity reactions. Here, we critically review the clinical and analytical tools used. In addition, we propose an explanation of the immune-mediated side-effects of PEGylated products based on the haptogenic properties of PEG, responsible for complement activation and the induction of anti-PEG antibodies.

Introduction

Advances in biotechnology and drug delivery research have produced many novel protein and liposomal therapeutics and hold the promise of even more-innovative products for areas in which few or no therapies exist. Despite these advances, many of these therapeutics have several shortcomings that limit their clinical utility, such as short circulation times, direct toxicity, rapid renal clearance and propensity to induce antidrug antibodies [1]. To overcome these shortcomings, many strategies have been applied to protein therapeutics such as changing the amino acid sequence or conjugation to a fusion protein. Although useful in certain applications, these methods also have their own limitations such as reduced affinity and activity and a higher tendency to aggregate [2,3].

Another strategy that has been investigated extensively is the attachment of polymers to therapeutic proteins. The polymer polyethylene glycol (PEG), a linear and nonionic polyether diol with the molecular formula $HO–(CH_2–CH_2–O)_n–H$, has been used extensively in the development of drug delivery systems as well as for protein modifications since its introduction in the 1970s. Its use in protein modification was first described by Abuchowsky *et al.* who showed that by PEGylation – the covalent attachment of PEG to form a conjugate – a protein could be modified extensively while maintaining its biological activity. They reported that with increased levels of attached PEG the immunogenicity of bovine serum albumin (BSA) and liver catalase can be reduced in rabbits [4,5]. The authors also showed that, in contrast to native liver catalase, clearance and immunogenicity of PEGylated catalase remained unchanged after multiple injections [5]. These findings ultimately led to a whole new discipline in drug development that has resulted in the marketing authorization of several PEGylated proteins including four blockbusters: PegIntron® (Schering-Plough, USA), Pegasys® (Hoffmann-La Roche, USA), Neulasta® (Amgen, USA) and Mircera® (Hoffmann-La Roche, USA).

PEGylation is also extensively applied in other fields of drug delivery research to increase serum half-life of therapeutics [6]. An example is PEGylated liposomes referred to as 'stealth liposomes', which accumulate less extensively in the liver than their non-PEGylated counterparts [7]. However, despite the so-called stealth properties of PEGylated liposomal therapeutics, Doxil® (Johnson and Johnson, USA; Schering Plough, Europe) is currently the only approved PEGylated liposomal drug delivery system that has reached the market [8].

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In addition to a large molecular size, the beneficial properties of PEGylation are also caused by the ability to attract a water shell around the polymer because each ethylene glycol subunit attracts two or three water molecules [1]. The attraction of water can increase the hydrodynamic size by a factor of five-to-ten, attenuating rapid renal clearance of therapeutics. In addition, PEGylation via steric hindrance shields the drug component from enzymatic degradation and opsonization with serum proteins, the binding of certain proteins that interact with the immune system [1,9]. Consequently, PEGylation can reduce uptake by the mononuclear phagocyte system (MPS), an obstacle that has hindered the wide introduction and application of many therapeutic proteins [1,10–12]. As a result, PEGylated therapeutics are thought to be more stable, induce fewer adverse events and have improved pharmacokinetics compared with their non-PEGylated counterparts [1].

However, in contrast to this, several papers from the past decade suggested that PEG – therapeutics are actually capable of inducing anti-PEG antibodies involved in rapid clearance of a subsequent dose [8,10,13]. Although the immunogenicity of PEG has not gained much attention in the field of protein therapeutics, the potential negative side-effects of PEG in other drug delivery systems have gained widespread attention [11,14]. Several research groups have shown that anti-PEG antibodies are associated with the rapid clearance of subsequent doses of PEGylated liposomes and micelles, referred to as the accelerated blood clearance (ABC) phenomenon [10,15,16]. In addition, several animal studies as well as clinical observations with the use of Doxil® have shown that PEGylated liposomes can activate the complement system and potentially cause hypersensitivity reactions [14,17,18]. Because PEGylation of therapeutics is currently considered to be one of the most favorable approaches in reducing immunogenicity and obtaining favorable pharmacokinetics, the existence and implications of anti-PEG antibodies as well as PEG-induced complement activation require thorough investigation.

Clinical symptoms and implication of PEGylated therapeutics

Although the immunogenicity of PEG is gaining increased attention, it should be noted that several PEGylated therapeutics have reached the market, most of which have not been linked to serious immunogenicity issues. For instance Pegintron®, which is now the standard treatment option for hepatitis C virus (HCV) infection, induces antidrug antibodies in ~10% of patients, but only results in diminished clinical outcome in ~1% of all patients [19]. It is therefore not surprising that many question the immunogenicity of PEG. Indeed, besides PEGylated liposomes only a few therapeutics have been associated with anti-PEG antibodies, such as PEGloticase (Krystexxa®, Savient, USA) and PEGasparginase (Oncaspar®, Ovation, USA) [20]. Importantly, PEGinesatide (Omontys®, Affymax and Takeda, USA) was recently withdrawn from the market owing to severe hypersensitivity reactions with even fatal consequences, which could be related to PEG [21,22]. Although anti-PEG antibodies and PEG-induced complement activation have only been studied by a few research groups and some PEGylated therapeutic proteins have been used apparently safely for several years, one should realize that PEGylation can result in a conjugate that is highly immunogenic. Indeed, one of the explanations why the immunogenicity of PEG has not obtained much attention is that most studies have only investigated immune responses against the active protein and therefore any immunogenicity issues most often have been attributed to the therapeutic itself [23,24]. Although the study of anti-PEG can be overlooked by researchers owing to the perception that PEG is biologically inert, analysis of anti-PEG antibodies is also extremely difficult as mentioned in a previous review paper by our group [25]. This is mainly caused by the fact that no available assay has been thoroughly investigated for its sensitivity and specificity. In addition, standards are often not well described or their extrapolation to titers measured in the assays. This also includes the commercially available double-antigen bridging ELISA described by Liu and colleagues [26]. Essential information is not provided such as the specificity and characterization of their monoclonal ANPEG-1 mouse IgM antibody used for quantification of samples and quality control [25]. As such, these assays can only be interpreted as semiquantitative. Although a validated assay format is not available, the FDA recently updated their guidelines to screen for anti-PEG antibodies during (pre)clinical trials as a precautionary measure.

The first report concerning an immune response against the PEG portion of a PEGylated protein was published in 1983 by Richter and Akerblom, who observed rapid clearance of a second dose of PEGylated ovalbumin in mice was related to increased levels of anti-PEG IgM antibodies [27]. One year later, the authors were also the first to report the induction of anti-PEG IgM in humans [28]. They reported that 50% of patients being treated with PEGylated ragweed extract and PEGylated honeybee venom developed anti-PEG antibody titers after their first dose. However, in contrast to their findings in mice, no changes in response rates were observed within the two years of treatment and the percentage of patients testing positive for anti-PEG decreased to 28.5% [28]. Unfortunately no information regarding the assays is available. The authors concluded that the anti-PEG antibodies were not of clinical significance because they can bind to PEG but do not neutralize the compound activity due to phagocytosis by macrophages of the MPS [28]. These findings played an important part in establishing the perception that PEG is nonimmunogenic, which contributed to the widespread use of PEG in drug development.

Liposomal doxorubicin

The PEGylated liposomal formulation of doxorubicin Doxil® is being used for the treatment of ovarian cancer and AIDS-related Kaposi's sarcoma. Although Doxil® reduces the cardiac toxicity observed with free doxorubicin, Doxil® itself also has some serious adverse effects such as the hand–foot syndrome and high incidence of infusion reactions [29]. These infusion reactions are thought to be caused by the complement system [30]. Hypersensitivity reactions (HSR) have been reported in up to 25% of patients in some studies, with an average of 8% across all published studies [30].

Although Doxil® is known to induce complement activation it has never been associated with the ABC phenomenon. Indeed, only PEGylated liposomes without cytostatic drugs have been shown to be associated with the ABC phenomenon. Dams *et al.* were the first to report that a second dose of empty PEGylated liposomes was rapidly eliminated when injected 5 days later [15].

The ABC phenomenon of empty PEG-liposomes has since been extensively studied in animals and revealed a negative correlation between the dose and lipid content on the clearance rate; meaning that the higher the dose and/or lipid content of the first administration the lower the ABC phenomenon and anti-PEG IgM production [31]. By contrast, encapsulation of doxorubicin into PEGylated liposomes induced a substantial reduction in the amount of anti-PEG IgM and a concomitantly reduced complement activation in the sera of rats [32]. It is assumed that cytotoxic agents released from the liposomes accumulating in the spleen impair the production of anti-PEG IgM as a result of inhibition of B cell proliferation and/or killing of B cells in the marginal zone (see below) [11,33].

PEGinesatide

The FDA approved peginesatide (Omontys®, Affymax and Takeda, USA) in December 2011 for the treatment of anemia in adult patients with chronic kidney disease (CKD) on hemodialysis [34,35]. Peginesatide, a dimeric peptide that is covalently attached to PEG, was the first erythropoietin-stimulating agent (ESA) that had no structural similarity to recombinant human erythropoietin (rhuEPO) and did cross-react with anti-rhuEPO antibodies [22]. Although clinical trials concluded that the product was nonimmunogenic, peginesatide was voluntarily withdrawn from the market in February 2013 owing to serious HSR; including anaphylaxis which was life-threatening and in some cases fatal [21]. Fatal reactions occurred in ~0.02% of patients within 30 min of the start of their first intravenous administration [21]. Interestingly, no cases of HSR were observed for patients who obtained multiple doses or completed their dialysis session [21]. No patient-related data have been published explaining the mechanisms behind the HSR. However, the fact that all reactions occurred during the first dose suggests a potential role for non-antibody-mediated complement activation. Although this view is in contrast to the general perception of acute HSR, recent studies have confirmed that PEGylated therapeutics are indeed able to induce complement activation that is not triggered by antibodies directly (see below) [30,36]. By contrast, because no immunogenicity data have been published, the presence of any cross-reactive antibodies in these patients cannot be excluded.

PEGylated asparaginase

The enzyme asparaginase is used as a therapeutic protein for the treatment of acute lymphoblastic leukemia. In contrast to normal cells, these tumor cells are unable to synthesize the nonessential amino acid asparagine [37]. Asparaginase catalyzes the conversion of asparagine and glutamine into aspartic acid and ammonia, thereby depriving leukemic cells of circulating asparagines [37,38]. To reduce the immunogenicity of asparaginase, PEGylated asparginase (PEG-ASNase) (Oncaspar®, Ovation, USA) was developed in an effort to mask antigenic epitopes.

Armstrong *et al.* reported that in pediatric patients treated with PEG-ASNase for acute lymphoblastic leukemia 32% developed anti-PEG antibodies as measured by serology and 46% as determined by a flow-cytometry-based assay [20]. Furthermore, anti-PEG antibody titers, mainly IgM, were closely related to the rapid clearance of subsequent doses [20]. Surprisingly, 13% of patients treated with unmodified ASNase in the same study were also positive for anti-PEG antibodies, but no association was observed between serum ASNase activity and anti-PEG antibody levels [20].

The occurrence of anti-PEG antibodies in patients with no history of treatment with a PEGylated therapeutic was also addressed by the same authors. They showed that 25% of healthy volunteers who had never been treated with a PEGylated drug had anti-PEG antibodies in serum. Their assays were based on agglutination of mPEG-SPA red blood cells and also flow cytometry using Tenta-Gel-OH beads grafted with 60–70% PEG chains [39]. This high percentage is explained by some researchers as a result of the large amount of PEG that is present in cosmetics and food products, which might actually induce anti-PEG antibodies [8]. However, this high percentage could be questioned despite a study by Tillmann *et al.* who reported the occurrence of anti-PEG antibodies in 44% of patients with HCV before treatment with PEG–interferon (IFN), no other clinical trials have reported anti-PEG antibody titers in healthy patients [40].

PEGylated uricase

In September 2010, PEGylated uricase (pegloticase) (Krystexxa®, Savient, USA) was approved by the FDA for the treatment of gout, a purine metabolic disorder that results in urate deposition in tissues and can lead to arthritis [41]. Unfortunately, the unpegylated enzyme is a foreign protein for patients and highly immunogenic because humans do not express this enzyme [42].

Response rates of pegloticase in two Phase III clinical trials were between 38% and 47% in the biweekly group and between 20% and 49% in the monthly group [23]. Anti-pegloticase and anti-PEG antibodies were detected in 88% and 32%, respectively, of subjects, and titers were correlated with the rapid clearance of pegloticase [43]. Of the anti-pegloticase-positive patients, 78% had IgG and IgM antibodies, 20% only IgM and 2% were only positive for IgG [43]. In addition, patients having IgG and IgM antibodies normally had the highest levels, and IgM only titers usually preceded that of IgG [43]. Anti-pegloticase and anti-PEG antibodies were detected using ELISA wells coated with either pegloticase or PEG. Anti-pegloticase antibodies bound other PEGylated proteins, including PEG–asparaginase, PEG–catalase, PEG–chymotrypsin and PEG–subtilisin as well as PEG–superoxide-dismutase, from which the authors concluded that antipegloticase antibodies were primarily directed against the PEG portion of the protein [43,44]. Although the authors reported that these antibodies were unable to reduce activity in their *in vitro* neutralizing assay, a strong correlation between rapid clearance, antipegloticase antibodies and complement activation were reported [23,43].

A strong relation between infusion reactions, high antipegloticase titers and complement activation was also observed [43,45]. Furthermore, more than 50% of transient responders who had activated complement appeared to have one or more signs of an infusion reaction of which only a small portion could be explained by IgE antibodies [23,43]. Because mainly IgM antibodies against pegloticase developed in these patients, a potential role of complement activation responsible for these infusion reactions cannot be excluded. It is well known that patients with gout can have activated complement levels owing to high levels of urate crystals [46]. When urate crystals activate the complement system, binding of IgM to pegloticase can further activate the complement systems at lower binding levels as a result of synergetic properties

and this could explain why infusion reactions are more common in patients with high IgM antibody titers.

The specificity of the antipegloticase antibodies remains however uncertain. Approximately 88% of attenuated responders developed antipegloticase antibodies that were able to cross-react with the PEG portion of other PEGylated proteins. However their specific anti-PEG ELISA only detected anti-PEG antibodies in 32% of patients. This could be explained by the much lower assay sensitivity of the anti-PEG ELISA in which PEG was coated to the plate compared with the antipegloticase assay, but it underpins the problems with anti-PEG ELISA assays as recently mentioned by Schellekens and colleagues. They state that current assays are not well validated for their specificity because of the absence of positive controls and well-defined dose–response curves [25]. In addition, the data on the specificity and affinity of anti-PEG antibodies in the anti-PEG assay raise questions because antibodies measured in the anti-PEG competition ELISA had higher affinity toward pegloticase than to free PEG polymers [43]. We therefore propose two possible mechanisms by which anti-PEG antibodies can bind to the PEG portion of a therapeutic. The first being that anti-PEG antibodies are directed against the PEG protein conjugation site and not to the PEG chain solely, as also suggested in other studies [10]. Unfortunately no data were published regarding the assay design, but binding in the anti-PEG ELISA could be explained by binding to PEG and the linker between the PEG and the well plate. It is possible that PEG itself is indeed not immunogenic but instead behaves as a hapten, meaning that it can become immunogenic when bound to a specific conjugate (see below). The second hypothesis is that anti-PEG antibodies are actually antipegloticase antibodies that cross-react with PEG. Because anti-PEG antibodies were only present in patients with high antibody titers and only appeared after antipegloticase antibodies were developed, it is possible that antipegloticase antibodies possess cross-reactivity toward PEG with lower avidity than to pegloticase [43]. The ELISA data of pegloticase are often referred to in discussions around anti-PEG antibodies. Owing to the poorly defined ELISA and the fact that the native protein is highly immunogenic, it is warranted to underpin the argument that PEG itself is able to induce an immune response by this example.

PEGylated IFN therapeutics

IFNs are a family of naturally occurring proteins produced by cells of the immune system. IFN therapeutics are clinically used for the treatment of: viral infections such as hepatitis B virus (HBV) and HCV; the primary treatment for several malignancies, such as melanoma, hairy cell leukemia and non-Hodgkin's lymphoma; and the treatment of autoimmune diseases such as multiple sclerosis [47]. Although these treatments are very effective, the percentage of non-responders is relatively high. The immunogenicity of these therapeutics has therefore been studied extensively [48].

Currently, two PEGylated IFN products have gained marketing authorization: PEGylated IFN alfa-2b (Pegintron®, Schering-Plough, USA) for the treatment of HCV; and PEGylated IFN alfa-2a (Pegasys®, Hoffmann-La Roche, USA) for the treatment of HBV. Both products are considered to have relatively longer circulating times and decreased immunogenicity than their non-PEGylated counterparts [49,50]. However, overall effectiveness remains relatively low for both products with response rates ~50% [1,49]. The number of studies reporting immunogenicity data is limited, and little research has been performed to determine the specificity of these antibodies. Most studies were performed using ELISA against either PEG–IFN or IFN [24,51] and only a few studies have investigated the potential existence of anti-PEG antibodies. For instance, Tillmann *et al*. evaluated the development, frequency and impact of anti-PEG antibodies in patients with HCV before and after treatment with either PEG–IFN-a2a or PEG–IFN-a2b. Anti-PEG antibodies were tested with an ELISA well coated with either pegloticase or 10 kDa PEG, and by competition with free PEG [40]. Anti-PEG antibody prevalence before therapy was 44% in HCV patients, which was significantly higher than the 6.9% in healthy controls [40]. However, the authors concluded that anti-PEG antibodies did not result in impaired response because sustained antiviral response was still achieved in 60% of anti-PEG-positive patients [40]. In 2010, a PEGylated INF-beta-1a (PEG–IFN-b1a, Biogen, USA) was investigated in two clinical Phase I trials for the treatment of relapsing multiple sclerosis. The study showed response rates of ~40%, and none of the subjects developed antibodies or neutralizing antibodies (NABs) against the IFN-b1a portion in the multiple dose group. By contrast, 8% of patients developed anti-PEG antibodies after a single dose [52].

Despite PEGylation, Pegintron® and Pegasys® can still induce antidrug antibodies just like their non-PEGylated counterparts. The clinical implications of this immunogenicity are unknown, and unfortunately only a few studies examined the specificity of these antibodies to be directed against the PEG chain [24,53]. Because these two products are both blockbusters and more PEGylated IFN products will enter clinical trials, it is of great importance to study the potential induction of anti-PEG antibodies and their clinical impact. Besides, no articles were found that studied the interaction of antibodies with the immune system in detail. Indeed, only binding capacity was assessed by determining antibody titers using ELISA and their neutralizing capacity in HCV-replicon IFN assays. However, many studies reported that anti-PEG antibodies can induce endocytosis by phagocytic cells owing to the activation of the complement system. Because it is hypothesized that non-NABs can further activate the immune system, *in vitro* and *in situ* complement activation studies as well as *in situ* phagocytic uptake studies deserve more attention [16].

Mechanisms of immunogenicity

Although PEGylated therapeutics are generally less immunogenic than their non-PEGylated counterparts, many clinically approved PEGylated therapeutics can still induce an immune response in a significant fraction of patients. The exact immunogenicity mechanisms are unknown, and little is known regarding a potential immune response against the PEG portion in PEGylated protein therapeutics. However, extensive research regarding the immunogenicity of PEGylated liposomes has been performed, which is described below.

Innate immune response by splenic B cells

Several groups have elucidated that anti-PEG IgM antibodies, produced by the spleen in response to an administered first dose of PEGylated liposomes, are responsible for the rapid clearance of a second dose administered several days later [16,54,55]. Production

of anti-PEG IgM is believed to occur in the spleen. Several studies have shown that splenectomized mice do not produce anti-PEG IgM nor do they clear a second administered dose of PEGylated liposomes [11,13,31,55]. Anti-PEG antibodies are elicited without the stimulation of helper T cells because BALB/$c^{nu/nu}$ (T-cell-deficient) mice are capable of producing anti-PEG IgM [55]. These results provide strong evidence that anti-PEG IgM is secreted by splenic B cells by a mechanism that is not dependent on T cells.

Although the follicle region in the spleen is the main compartment for B cells, several studies have shown that PEGylated liposomes mainly bind B cells in the marginal zone [11]. It is thought that upon stimulation the marginal zone B cells (MZ B cells) rapidly proliferate and differentiate into either antigen-presenting cells or into IgM-secreting plasma cells [11]. Production of anti-PEG IgM behaves in a wave pattern, meaning that after the first injection anti-PEG IgM titers increase at day 3, peak at day 5 and then gradually decrease until undetectable at day 28 [13,16,33,56]. Indeed, Li *et al.* showed that intravenous administration of PEGylated liposomes in beagles only resulted in the ABC phenomenon when a second dose was administered within 3 weeks. A second dose was not rapidly cleared when the time interval was prolonged to 4 weeks [57].

Because immune memory is not established, antibody production seems to be mediated by the innate immune system. This is one of the evolutionarily older systems that can produce antibodies without the help from T cells in response to pathogen-specific antigens consisting of repeating structures, such as lipopolysaccharides on the cell walls of Gram-negative bacteria [58]. The innate immune response is thought to be due to two types of thymus-independent (TI) B cells, depending on co-stimulating factors and the ability to cross-link [59]. A type 1 response occurs when B cells bind to an antigen and receive secondary activation by Toll-like receptors, and a type 2 response occurs when enough B cells can simultaneously cross-link antigens [11,59,60]. Owing to the multireactivity properties of B cells and their strong response to membrane-associated antigens, B cells probably recognize the PEG conjugate in a type 2 manner owing to its composition of multiple repeating structures [61].

Antibody classification and antigenic determinant

Anti-PEG antibodies have been found to be mainly IgM, especially in the studies identifying antibodies responsible for the rapid clearance of PEGylated liposomes [32,55,61,62]. However, most studies make use of an ELISA format in which lipids such as DSPE–PEG are being used as the antigen. The exact specificity of anti-PEG antibodies is still unknown; but, as mentioned above, several studies have shown that anti-PEG antibodies produced in response to a certain PEG – therapeutic can also bind other PEGylated therapeutics *in vitro* and *in vivo* [43,62,63]. In addition, Armstrong reported that the antigenic determinant of anti-PEG antibodies found in 25% of healthy volunteers was directed toward the PEG chain. Agglutination in the red cell assay was completely inhibited by PEG polymers sized from 300 to 20,000 Da, polypropylene glycol 2000 Da and tri- and tetra-(ethylene glycol)dimethyl ether and penta(ethylene glycol) [64]. From these results they concluded that the minimum epitope of recognition by anti-PEG antibodies is 4–5 repeated oxyethylene units [64].

Although this result might imply that PEG itself is the immunogenic determinant, several studies have shown that a single injection of PEG polymers alone does not induce an anti-PEG immune response [11,27]. This phenomenon is explained by the hypothesis that the production of antibodies only occurs against PEG conjugates in a haptogenic manner; meaning that PEG only elucidates an immune response when conjugated [10,28,65]. The haptogenic properties of PEG fit well with the clinical data and depend on its molecular weight, the immunogenicity of the conjugated protein or nanocarrier, the presence of adjuvants and potentially by the terminal end-group of PEG [27,28,65,66]. Besides, it is thought that MZ B cells possess a stronger response to membrane-associated antigens than to soluble antigens [11,67]. In addition, the conjugate itself does need to be immunogenic to induce a haptogenic immune response. The literature implies that, although PEG could be the antigenic determinant for anti-PEG antibodies based on their cross-reactive properties, anti-PEG antibodies can only be induced when the PEG conjugate behaves as a hapten.

Anti-PEG antibodies versus dose

The induction of anti-PEG IgM and the subsequent accelerated clearance of PEGylated liposomes seem to have an inverse relationship with the quantity of the first dose. The same relationship is seen for the accumulation of PEGylated nanocarriers in the marginal zone of spleen after a second dose. More PEGylated nanocarriers associate with marginal B cells when a low first dose was administered compared with a high first dose [16]. This observation implies that an optimal amount of TI-2 antigens need to cross-link with B cells to induce an antibody response. It is proposed that, when a high dose is administered as a first dose, the density of TI-2 antigens is too high and this causes splenic MZ B cells to induce immune tolerance or anergy [11]. In addition, it has been shown that subsequent receptor signaling is needed to maintain anergy of B cells [68]. Because higher doses lead to prolonged circulation, it is thought that PEGylated therapeutics are in increased contact with MZ B cells, which again can contribute to immune tolerance or anergy [16].

Complement activation

To summarize the above discussion, it is thought that PEGylated therapeutics can be recognized by splenic B cells, which are activated by a thymus-independent mechanism, as a result of cross-linking of multiple B cells to either PEG or the PEG–therapeutic conjugation site. As a result, these B cells produce anti-PEG IgM, which has been shown to correlate with the clearance of a second administered dose.

However, IgM antibodies are unable to promote phagocytosis directly because IgM is not an opsonizing antibody as a result of the absence of Fc receptors for IgM on the surface of macrophages [69]. Instead, binding of IgM can trigger opsonization of complement factors that subsequently promote phagocytosis by Kupffer cells bearing complement receptors [69,70]. Several of these complement factors are known to have an important role in HSR toward certain therapeutics. Indeed, clinical studies have linked elevated complement factors to infusion reactions in response to PEGylated proteins and nanocarriers such as PEGylated asparaginase and the liposomal formulation Doxil® [14,71].

Complement activation in response to PEGylated therapeutics

Indications that complement factors are involved in the ABC phenomenon by IgM antibodies arose when Dams *et al*. showed that rats that had undergone a transfusion of serum from rats pretreated with PEGylated liposomes were able to clear the dose rapidly when they were treated with PEGylated liposomes for the first time [15]. In addition, the phenomenon could be abolished by pre-heating the serum at 56°C for 30 min before transfusion, the temperature at which complement is deactivated [15,18].

Several studies have indicated that the molecular weight and concentration of PEG is a crucial factor for complement activation. For instance *in situ* studies by Shimizu *et al*. showed that PEG_{30000}–BSA induced higher anti-PEG IgM titers and greater complement activation than PEG_{2000}–BSA; and extensive research on masking red blood cells by PEG has shown that cell lysis caused by complement activation was actually enhanced with increased concentrations of PEG [16,72,73].

Non-antibody-mediated complement activation

In contrast to complement activation through the classical pathway, several studies have reported that PEGylated therapeutics can activate the complement system after a first dose [21,29,30]. Until recently, acute allergic reactions were believed to be mainly IgE-mediated as defined by the Gell and Coombs classification of HSR [36]. However, data from the past decade have shown that more than 75% of acute allergic reactions are actually not initiated or mediated by IgE antibodies, and are referred to as pseudoallergic reactions [74]. Although the exact mechanisms are not well understood, there is evidence that most of these HSR are triggered by complement activation and caused by anaphylatoxins [74]. However the role of cross-reactive IgM or IgG antibodies has never fully been investigated to exclude the influence of an antibody-mediated response in these pseudoallergic reactions.

Extensive research by Szebeni *et al*. has shown that complement activation has a causal relation with the infusion reactions observed in up to 25% of patients treated with Doxil®, which were non-IgE-mediated [29,71,74]. In contrast to the general perception, they have also shown that these reactions can occur after a first administration, which implies that Doxil® can directly activate the complement system by the mannose-binding lectin pathway or the alternative pathway [17]. Furthermore, vesicles of the same size and composition as Doxil® that did not bear doxorubicin were also able to trigger complement activation *in vitro* [75]. In addition, it was recently reported that highly concentrated free PEG or polysorbate 80 are able to generate complement activation in serum within minutes [14,76]. It remains however unclear whether complement activation in response to a first dose is able to induce phagocytosis besides the generation of anaphylatoxins, as Chanan-Khan *et al*. reported that the ABC phenomenon was not present in 92% of patients treated with Doxil® despite having elevated SC5b-9 levels [30]. It seems that phagocytosis only occurs with the presence of antibodies, which in the case of PEGylated nanocarriers containing cytostatic drugs does not occur as a result of its toxicity toward MZ B cells [31,77].

As extensive research on Doxil® implies that PEGylation can directly activate the complement system during a first dose, it is hypothesized that other PEGylated therapeutics can activate complement in a non-antibody-mediated manner. For instance, peginesatide has been withdrawn from the market owing to anaphylaxis, of which some cases resulted in death [21]. Unfortunately no hematologic data have been published, but the clinical symptoms as well as the fact that all cases occurred within 30 min of the start of the first administration suggests non-antibody-mediated complement activation [22].

Concluding remarks and recommendations

PEGylation is being applied in many different therapeutic fields because of its promising properties for increasing pharmacokinetics because of increased hydrodynamic size and shielding against immunogenic epitopes by means of steric hindrance against the MPS [1]. For instance, its shielding properties have enabled non-human proteins such as uricase and asparginase to be administered to humans. However, despite the belief that PEGylation can reduce immunogenicity of non-human proteins in human patients, a significant fraction of patients still produce antidrug antibodies. Immunogenicity against PEGylated proteins is often explained by an immune response against the protein component, but thorough studies investigating the specificity of these antibodies are rarely performed. Anti-PEG antibodies induced by PEGylated nanocarriers such as liposomes is a phenomenon becoming more accepted as the perpetrator of the ABC phenomenon. These antibodies are capable of rapidly clearing a subsequent dose injected a few days later. Although it appears that the production of these anti-PEG antibodies does not result in immunological memory, doses administered within 3–4 weeks of the first dose can still be cleared [56].

The FDA recently updated their guidelines to screen for anti-PEG antibodies during clinical trials of PEGylated therapeutics. Although several studies have implied that PEGylated protein therapeutics can induce anti-PEG antibodies responsible for an attenuated response, too few studies have investigated the specificity of these antibodies to conclude if anti-PEG antibodies can influence the pharmacokinetics of PEGylated proteins [16,43].

An important obstacle regarding immunogenicity studies is that no validated anti-PEG assay, including appropriate standards, has yet been developed. Few results have been published regarding the sensitivity and selectivity of these assays [25]. These deficiencies have led to a discussion among scientists about how to interpret anti-PEG antibody data, including potential cross-reactivity with other PEGylated therapeutics as well as polysorbates [64,78]. In addition, different results have been reported on the pre-treatment occurrence of anti-PEG in humans as well as the amount of anti-PEG produced in response to PEGylated therapeutics [23,43,65]. It is therefore recommended that a fully validated assay is developed by collaboration between different research groups as soon as possible. Because it is believed that anti-PEG antibodies are developed against the PEG chain and its conjugate, the biggest challenge will be to develop a standard that can be used to analyze samples from patients treated with different products. Anti-PEG antibodies developed in patients treated with pegloticase also bound other PEGylated proteins. Although the affinity of these antibodies toward these products was not mentioned, it provides some hope that a commercial mono- or poly-clonal anti-PEG standard can be developed. It is therefore highly recommended that parties developing such an assay show specificity and affinity data toward different kinds of PEGylated products.

It is also recommended to analyze the activation of complement thoroughly *in vitro* and *in vivo*. Because it seems that mainly anti-PEG IgM is produced against the PEG–therapeutic conjugation site, another component of the immune system needs to be involved because IgM antibodies cannot induce phagocytosis directly [69]. Until a validated anti-PEG assay is developed, studying complement activation in patients might provide an alternative method to gain insight into the immunogenicity and HSR caused by PEGylated therapeutics.

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