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Poly(ethylene glycol)s generate complement activation products in human serum through increased alternative pathway turnover and a MASP-2-dependent process

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ABSTRACT

Poly(ethylene glycol) (PEG) is receiving increasing attention as an intravenous therapeutic agent *per se* in a variety of experimental therapeutics and veterinary settings, such as spinal cord injury and traumatic axonal brain injury. PEG is often perceived to be immunologically safe, but here we demonstrate that near-monodisperse endotoxin-free PEGs, at concentrations relevant to above-mentioned settings, can generate complement activation products in human serum on a time scale of minutes (reflected in significant rises in serum levels of C4d, Bb, C3a-desArg and SC5b-9). With the aid of sera depleted from either C2 or C1q, and devoid of anti-PEG antibodies, we further demonstrate that, depending on PEG concentration and M_{wt}, generation of complement activation products occur either exclusively through the lectin pathway activation or through both the lectin pathway and increased fluid phase turnover of the alternative pathway. Inhibition of PEG-mediated C4d elevation in C1q-depleted serum by the broad serine protease inhibitor Futhan and anti-MASP-2 antibodies as well as competitive studies with D-mannose and *N*-acetylglucosamine indicated a likely role for ficolins/MASP-2 in PEG-mediated triggering of the lectin pathway and independent of calcium. PEG-mediated amplification of the alternative pathway is a complex process related to protein partitioning and exclusion effect, but factor H depletion/exclusion seems to play a minor role. Our results are relevant to the proposed potential therapeutic applications of intravenous PEG and warn about possible acute PEG infusion-related reactions in sensitive individuals and animals. PEG-mediated generation of complement activation products further provides a plausible explanation to the previously reported unexplained anaphylaxis or the referred cardiovascular collapse in sensitive animals that have received medicines containing high levels of PEG as solubilizer/carrier.

1. Introduction

Poly(ethylene glycol) (PEG) is a linear polyether diol [HO–(CH_2–CH_2–O)$_n$–H where n is the degree of polymerization]. This non-ionic surfactant, which is commonly used in a wide range of intravenous human and veterinary pharmaceutical formulations, has so far been perceived to be immunologically safe (although it can be immunogeneic), and is eliminated from the body intact by the kidneys (for PEGs > 20,000 g mol^{-1}) (Yamaoka et al., 1994; Harris and Chess, 2003). More recently, PEG was identified as a therapeutic agent *per se* in a variety of experimental therapeutics and veterinary settings (Laverty et al., 2004; Koob and Borgens, 2006). A remarkable example is the ability of intravenously injected PEG_{3500}, particularly at high doses (600 mg/kg,), to repair acute, naturally occurring paraplegia in dogs (Hansen type 1 lessions) (Laverty et al., 2004). This experiment translated to an infusion dose of 13 mg PEG per second for 15 min to a 20-kg dog. PEG is believed to target the spinal cord contusion and "anatomically" seal the membranes of damaged axons through membrane fusion and restore excitability. Indeed, earlier, direct application of PEG to exposed contusion injuries in guinea pigs was shown to rapidly restore variable levels of nerve impulse conduction through the lesion, as documented by a rapid recovery of both somatosensory evoked potential conduction and cutaneous trunchi muscle reflex (Borgens and Shi, 2000). Also, a recent study has further demonstrated that following traumatic axonal brain injury in rats, intravenously injected PEG can enter the brain parenchyma and repair cell membrane damage in corpus callosum

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and eliminate β-amyloid precursor protein accumulation in the region of injury (Koob and Borgens, 2006).

Remarkably, the data sheet on some high PEG content intravenous medicines (e.g., as in the veterinary arena) warns about unexplained acute adverse reactions such as ataxia, restlessness and trembling, respiratory abnormalities, frothing at the mouth, collapse and even death in some cattle, sheep and swine receiving such formulations. Interestingly, adverse non-IgE-mediated hypersensitivity reactions, which are also associated with cardiac anaphylaxis and rapid haemodynamic collapse, are known to occur in some humans and animals who have received intravenous formulations containing macromolecular non-ionic surfactants that are structurally similar to PEG. Examples include polyethylene oxide–polypropylene oxide-based block copolymers, such as poloxamer 188 (Tremper et al., 1984; Police et al., 1985; Kent et al., 1990). Block copolymers like poloxamer 188 trigger complement activation both at sub-micellar concentrations as well as in micellar forms (Moghimi et al., 2004), and the observed adverse responses following administration of poloxamer-based medicines are strongly believed to be secondary to complement activation through the generation of anaphylatoxins C3a and C5a, leading to the subsequent release of thromobxane A2 and other inflammatory mediators from immune cells (Vercellotti et al., 1982; Ingram et al., 1993; Moghimi et al., 2004; Szebeni, 2005). In addition to these mediators the multiprotein terminal complex C5b-9, also has the capacity to elicit non-lytic stimulatory responses from vascular endothelial cells and further modulate endothelial regulation of haemostasis and inflammatory cell recruitment (Hattori et al., 1989).

In light of these observations, it is imperative to examine whether PEG can trigger complement activation, although it has long been considered to be a safe and a "biocompatible" macromolecule. For the first time, we demonstrate that near-monodisperse endotoxin-free PEGs, at concentrations relevant to the above-mentioned experimental scenarios, trigger complement activation in human sera. Depending on PEG concentration and M_{wt}, complement activation proceeds either exclusively through MASP-2 activation, and a likely role for the lectin pathway, or through both MASP-2-mediated C4 cleavage and accelerated alternative pathway turnover.

2. Materials and methods

2.1. PEG samples

Fluka GPC standard PEGs were obtained from Sigma–Aldrich (UK). The weight average molecular weight (M_{wt}), the number average molecular weight (M_n) and polydispersity index of PEG samples are presented in Table 1. The absence of endotoxin in all PEG solutions (in sterile saline) was confirmed by Limulus amebocyte lysate test.

2.2. PEG diacetylation

For mechanistic studies only PEG_{4240} was subjected to acetylation thus modifying the terminal OH groups. PEG_{4240} (100 mg) in pyridine (40 mL) and acetic anhydride (16 mL) was stirred at room temperature overnight. The mixture was poured into dilute hydrochloric acid and the product extracted with ethyl acetate. The combined extracts were washed with copper sulfate solution, aqueous sodium hydrogen carbonate, water, saturated sodium chloride solution and dried over sodium sulfate. The solvent was removed *in vacuo* to afford diacetoxyPEG as a gum. Acetylation of the PEG_{4240} was confirmed by both ^1H and ^{13}C NMR spectroscopy [($CDCl_3$; 360 MHz) ^1H NMR 2.08 ppm (—$COCH_3$) 3.64 ppm (—CH_2—); ^{13}C NMR 19.81 ppm (—$COCH_3$) 70.55 ppm (—CH_2—) 169.89 ppm (—$COCH_3$)]. The ^1H NMR of the starting material contained a signal at 3.64 ppm consistent with CH_2 in the polymer backbone. In addition to this resonance the product contained a new peak at 2.08 ppm from the terminal methyl group of the acetate. Further structural support for the diacetylation was found in the ^{13}C NMR spectrum of the product with new methyl (19.81 ppm) and quaternary carbon (169.89 ppm) resonances present for the acetate group, but was devoid of the CH_2OH signal in the starting material thus confirming acetylation had taken place.

Table 1
PEG characteristics

Designation	M_{wt} (g mol^{-1})	M_n (g mol^{-1})	Polydispersity (M_{wt}/M_n)	$(OCH_2CH_2)_n$
PEG_{1960}	1,960	1,900	1.032	$n \sim 44$
PEG_{4240}	4,240	4,120	1.029	$n \sim 96$
PEG_{8350}	8,350	8,100	1.031	$n \sim 189$
PEG_{11600}	11,600	10,800	1.074	$n \sim 263$

2.3. Liposome preparation

Unilamelar vesicles of 204 ± 39 nm (polydispersity index = 0.065), and composed of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol) and cholesterol in a mole ratio of 50:5:45 were prepared by hydrating the dried lipid film with 10 mM phosphate-buffered saline (pH 7.2) and subsequent extrusion through polycarbonate Nuclopore filters of appropriate pore diameters using a high-pressure extruder (Moghimi et al., 2006). Liposome size was determined by dynamic laser light scattering, as described earlier (Moghimi et al., 2006).

2.4. Preparation of human serum

Blood was drawn from healthy Caucasian male volunteers (aged 25–35 years) according to approved local protocols. Blood was allowed to clot at room temperature and serum was prepared, aliquoted and stored at −80 °C. Serum samples were thawed and kept at 4 °C before incubation with test reagents (repeated freeze and thawing was avoided and frozen sera was used within 3 weeks). Commercially available human C1q-depleted serum and C2-depleted serum was obtained from Quidel (distributed by Technoclone, UK).

2.5. Assays of in vitro complement activation

To measure complement activation in vitro, we determined PEG-induced rise of serum complement activation product SC5b-9, C3a-desArg, Bb and C4d, using respective Quidel's ELISA kits according to the manufacturer's protocols as described previously (Moghimi et al., 2006; Hamad et al., 2008). As a result of substantial biological variation in serum levels of complement proteins and the large number of positive and negative feedback interactions (Hamad et al., 2008), we monitored generation of complement activation products in sera of five healthy individuals separately. The concentration of mannose binding lectin (MBL) in healthy and selected complement protein-depleted sera was determined by using the MBL-C4 complex ELISA kit (HyCult Biotechnology, The Netherlands) (Hamad et al., 2008). Only sera with physiological concentrations of MBL, in the range of 3000–5000 ng/mL, were selected for subsequent complement activation assays. The complement haemolytic activity of C1q-depleted serum and C2-depleted serum was restorable following the addition of C1q (180 μg/mL) and C2 (650 μg/mL), respectively as assessed by the

haemolytic test using sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody (Hamad et al., 2008). The functional activity of classical, lectin and the alternative pathways of complement were confirmed in all sera with Wielisa®-Total Complement Screen kit (Lund, Sweden) prior to PEG addition studies.

For measurement of complement activation, the reaction was started by adding the required quantity of PEG solutions (in sterile physiological saline) or liposomes to undiluted serum (PEG or liposome:serum volume, 1:4) in Eppendorf tubes (in triplicate) in a shaking water bath at 37 °C for 30 min, unless stated otherwise. Reactions were terminated by addition of "sample diluent" provided with assay kit. Control serum incubations contained saline (the same volume as PEGs or liposomes) for assessing background levels of complement activation products. Zymosan (Sigma–Aldrich, 5 mg/mL) was used as a positive control for complement activation. The level of the complement activation products was then measured by the respective ELISA kits and compared with control incubations in the absence of PEG. In some experiments, PEG-induced complement activation was monitored following pretreatment of serum with EGTA/Mg^{2+} (10 mM/2.5 mM), Futhan (150 μg/mL; Merck), anti-MASP-2 antibodies (HyCult Biotechnology, The Netherlands), an irrelevant murine IgG antibody, *N*-acetylglucosamine (25 mM), D-galactose (25 mM) and D-mannose (25 mM). Control serum incubations contained the same quantity of the added compounds and PEG was replaced with the same volume of saline. In all experiments the pH was maintained between 6.8 and 7.1 range.

For quantification of complement activation products, standard curves were constructed using the assigned concentration of each respective standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best-fit line for SC5b-9, C3a-desArg, Bb and C4d standard curves were within the manufacturer's specified range. The efficacy of PEG treatments was established by comparison with baseline levels using paired *t*-test; correlations between two variables were analyzed by linear regression, and differences between groups (when necessary) were examined using ANOVA followed by multiple comparisons with Student–Newmann–Keuls test. Similar patterns were observed in all tested sera; the result of a typical experiment is presented.

2.6. SDS-PAGE and Western blot analyses

Serum was treated with PEG_{4240} (5 mM and 10 mM final concentration) or PEG_{11600} (1.5 mM final concentration) for 5 min at room temperature. Aggregated proteins were precipitated by centrifugation (16,000 × *g*, 20 min) and re-suspended in physiological saline. Proteins were then subjected to SDS-PAGE using 10–12% gels, immunoblotted with a murine monoclonal antibody against factor H (1:2000, v/v; Quidel) and bound factor H was detected using horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000; HyCult). A commercially available factor H (Quidel) was used as a positive control.

3. Results and discussion

3.1. Activation of terminal pathway of complement

Activation of the terminal pathway culminates in the formation of the C5b-9 complex, which in the absence of a target membrane binds to vitronectin (S protein) (Moghimi et al., 2006). Therefore, PEG-mediated complement activation in human serum was first monitored by measuring the generation of the stable, soluble, non-lytic fluid phase SC5b-9 complex as a sensitive measure of the activation of the whole complement cascade. The results (Fig. 1a–d) demonstrate that PEGs of varying M_{wts} can activate complement in a concentration-dependent manner, but on molar basis high M_{wt} PEG species are more effective in activating complement than their low M_{wt} counterparts. The results (Fig. 1e) also show PEG-mediated anaphylatoxin (C3a-desArg) generation, providing additional evidence that PEG-induced rises of SC5b-9 levels in serum is a reflection of complement activation rather than modulation of the terminal pathway only. PEG-mediated SC5b-9 generation is C3-dependent as confirmed in C3-depleted human serum (not shown). However, in direct comparison to zymosan, PEGs are poor activators of the complement system.

3.2. Alternative pathway turnover

The results in Fig. 2a show that, on a molar basis, the longer the PEG molecules the more effective they are in triggering the generation of the alternative pathway split-product Bb (the results are expressed as fold increase for direct comparison). The role of C3 in PEG-mediated Bb generation was confirmed in C3-depleted serum (no significant elevation of Bb and SC5b-9 over background, not shown). These results may be interpreted on the basis that PEG acts directly on complement proteins (e.g., C3) or indirectly through water activity. Biophysical evidence has concluded that in an aqueous environment, PEG chains, as a result of hydrogen bond formation with water molecules, assume helical conformation, which follows *trans–gauche–trans* sequence (links across C—O—C—C, O—C—C—O, C—C—O—C bonds) (Crupi et al., 1996; Tasaki, 1996; Kozielski, 2006). These studies have further indicated that the extent of water clustering increases with PEG size; the hydration increases from two molecules of water per PEG monomer at very low polymerization (tetramer) to five molecules of water per PEG monomer for 45-mer. Generally, increasing PEG size and concentration (providing the PEG phase is not too ordered) both increases the proteins' effective hydration as the PEG is excluded from the protein's surface (Bhat and Timasheff, 1992). Indeed, previous studies have shown that a number of proteins (e.g., serum albumin and lysozyme) were preferentially hydrated in the presence of PEG (M_{wt} 200–6000), and the magnitude of hydration increased with increasing PEG size for each protein. In accordance with this phenomenon, a plausible effective C3 hydration and conformational changes in the presence of PEG may accelerate "C3 tickover", leading to the assembly of fluid phase C3bBb convertases. However, C3 partitioning (particularly through its non-polar surface residues) into hydrophobic PEG phase may further compete with the steric exclusion and partitioning will increase with PEG molecular mass (Bhat and Timasheff, 1992). This possibility may further contribute to amplification of the alternative pathway turnover and explain why longer PEG chains remain more effective than their shorter counterparts in triggering complement. Indeed, PEG_{1960} (Fig. 2a) is unable to elevate serum Bb levels even at concentrations as high as 20 mM (equivalent to 40 mg PEG/mL serum); presumably at such concentrations the PEG phase is too ordered resulting in exclusion of partitioned C3 due to the reduced available water content.

In a typical protein solution, PEGs, in a concentration-dependent manner, can eventually favour the formation of protein crystals by decreasing the protein solubility through "depletion attraction", but PEG can also induce other phase changes such as "liquid–liquid" phase separation, protein aggregation and the formation of gels (Asakura and Oosawa, 1954; Tardieu et al., 2002), which may dramatically affect the kinetics of complement activation. The results in Fig. 2b and c show that PEG_{11600}-mediated elevation of serum SC5b-9 and Bb levels proceeds on a time scale of minutes and

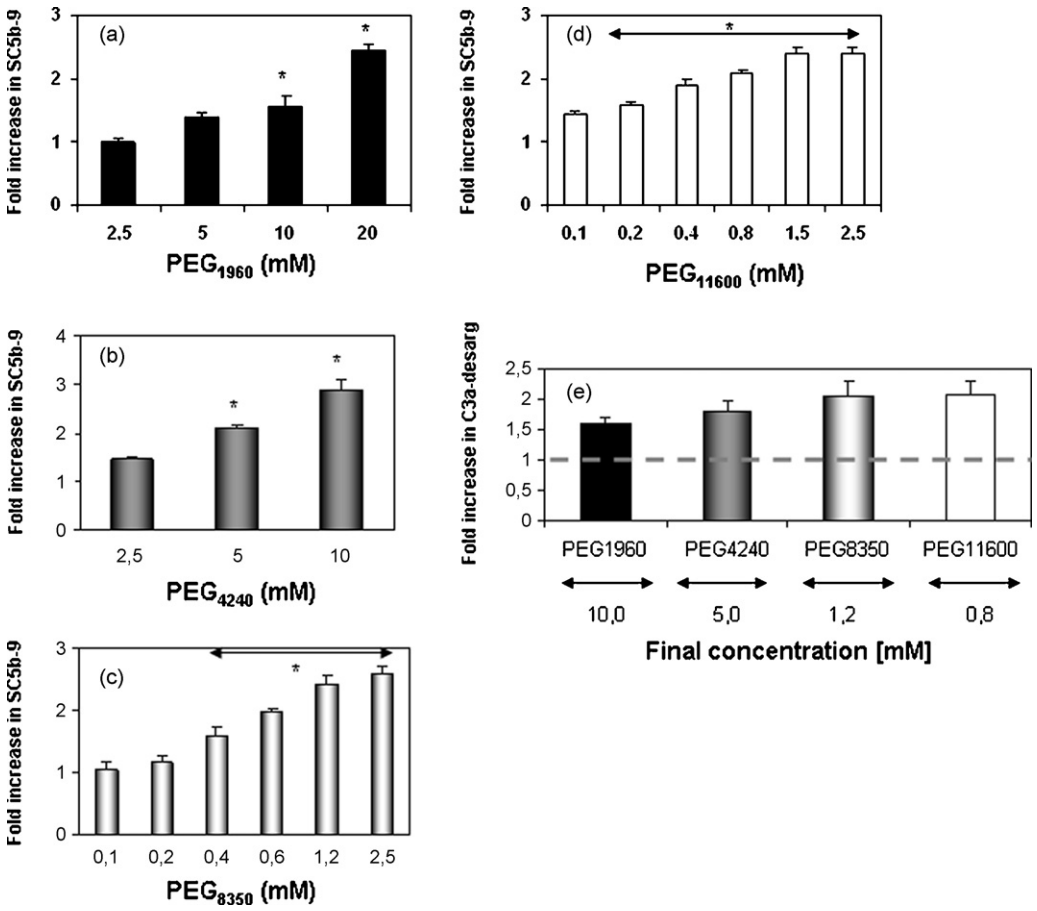

Fig. 1. Complement activation as a function of PEG concentration and molecular mass. PEG-mediated rises of SC5b-9 over background is represented in panels (a–d). The background SC5b-9 level in this particular serum (saline-treated) was 0.87 ± 0.05 μg/mL. Zymosan (5 mg/mL) was used as a positive control and raised serum SC5b-9 levels by 3.32-fold over background (2.90 ± 0.10 μg/mL). The influence of PEGs on anaphylatoxin C3a-desArg is shown in panel (e) and zymosan raised C3a-desArg levels by 3.4-fold over background. Similar patterns in SC5b-9 and C3a-desArg elevations were obtained in four other tested human sera. In (a–d) significant difference is expressed with respect to the background (saline-treated serum): $^{*}p < 0.05$. In panel (e) all values are significantly different from the background level ($p < 0.05$).

reaches plateau at about 5 min. These observations partially support the aforementioned arguments (rapid PEG backbone–protein interaction); complement activation presumably stops at 5 min as a result of rapid exclusion of some key partitioned proteins (arising from reduced available water content), such as C3. Similar profiles were also observed with PEG_{4240} (10 mM) and PEG_{8350} (not shown). Interestingly, such complement activation time scales are in line with the rapidity of the observed acute adverse reactions or cardiovascular collapse in sensitive animals receiving intravenous injections of PEG-containing medicines.

We further reasoned that PEG-mediated acceleration of the alternative pathway turnover may in part arise from partial depletion of factor H, the major fluid phase regulator of the alternative pathway (Pangburn et al., 1977). Factor H attenuates alternative pathway activation by inhibiting the binding of factor B to C3b, accelerating the decay of preformed C3Bb convertases and acting as a cofactor for the serine protease factor I to cleave C3b (Walport, 2001a,b). Western blot analysis of PEG_{4240}- and PEG_{11600}-induced protein aggregates in human serum has confirmed the presence of factor H (Fig. 2d). In the case of PEG_{11600}, the Western blot corresponded to 21 ± 5% of the total blotted factor H from untreated control serum under identical conditions at 5 min. With PEG_{4240} treatment, factor H was only detectable (17 ± 3%) when surfactant concentration was at 10 mM or above. It is rather unlikely that for such levels of factor H exclusion to play a significant role in controlling the rate of the alternative pathway turnover, but this could be a critical drive in enhancing fluid phase turnover in sera with low levels of factor H.

Acceleration of the alternative pathway turnover by high M_{wt} PEG species (M_{wt} = 4240 g mol^{-1} or 8350 g mol^{-1}) may further represent amplification of C3 convertases initially triggered through C4-dependent pathway (Lambris et al., 2008). In order to demonstrate that PEG molecules can directly enhance alternative pathway turnover we monitored Bb and SC5b-9 generation simultaneously in a C2-depleted human serum (Fig. 2e). With PEG_{8350} serum levels of both complement markers were significantly above the background (Fig. 2e), thus showing the capability of these species to directly affect alternative pathway turnover. On the contrary, PEG_{4240} only at 10 mM concentration was able to trigger Bb generation in C2-depleted serum (Fig. 2e). Although, at lower PEG_{4240} concentrations (5 mM) neither serum Bb nor SC5b-9 levels were elevated, significant rises in SC5b-9 levels above background in C2-depleted serum was only demonstrable following restoration of C2 at physiological concentrations (data not shown); this observation strongly suggest a role for the C4-dependent pathway and involvement of C4b2a convertases (since SC5b-9 generation was C3-dependent, see Section 3.1.) in subsequent assembly of the terminal complement complex.

We next established that acceleration of alternative pathway turnover was not due to the presence of possible PEG oxidation products as no signal in the aldehyde range was detected in both ^{1}H and ^{13}C NMR spectra, and in addition, the IR spectra of PEG samples

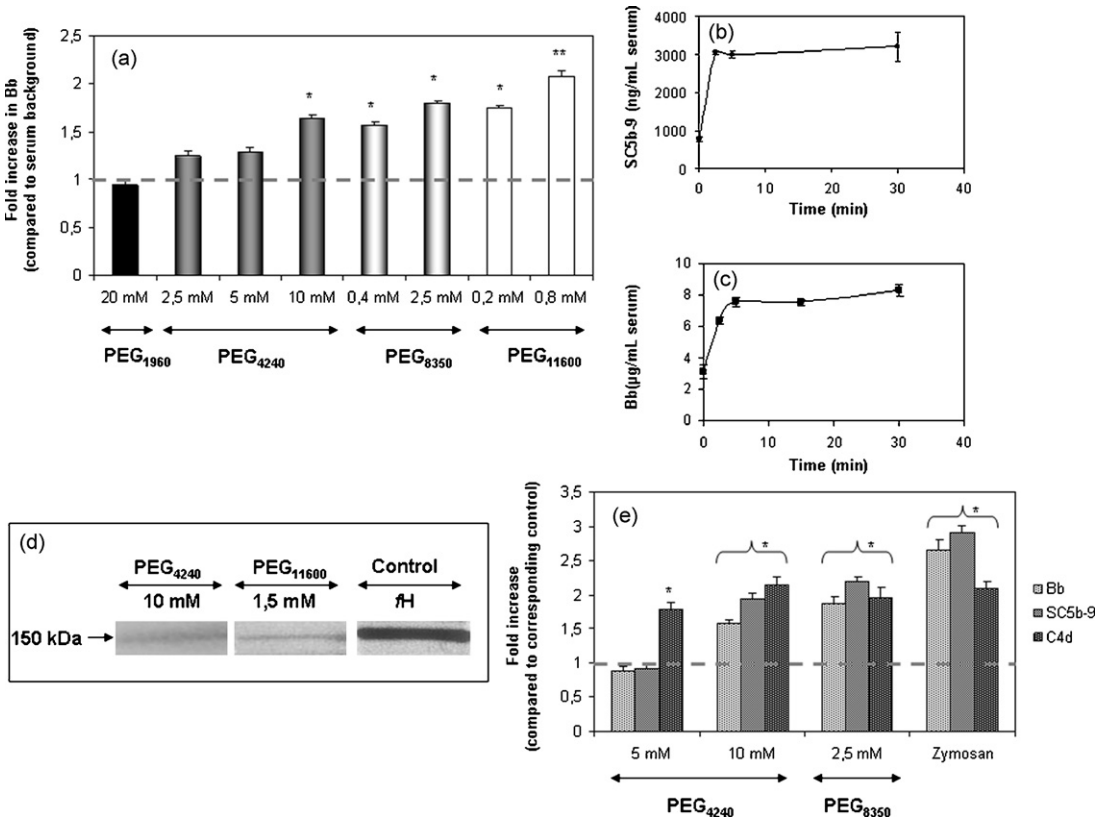

Fig. 2. PEG-mediated amplification of the alternative pathway turnover. Panel (a) shows the effect of PEG molar concentration and molecular mass on serum levels of the split-product Bb (the background Bb level was 0.42 ± 0.06 μg/mL). Serum source was the same as in Fig. 1. Similar patterns were further obtained with four other sera from separate individuals (not shown). Zymosan elevated serum Bb level by 3.66-fold, over background. Significant difference with respect to background (saline-treated serum): $^{*}p < 0.05$, $^{**}p < 0.01$. Panels (b) and (c) show kinetics of PEG_{11600}-mediated SC5b-9 and Bb generation in a normal serum. PEG concentration was 1.5 mM. Panel (d) is Western blot analysis of PEG-induced protein aggregates for factor H in normal serum. A commercially purified factor H was included as a positive control with immunoblots. In (e) PEG-mediated complement activation products are measured in a C2-depleted human serum. SC5b-9, Bb and C4d background levels (saline-treated serum) were 1.83 ± 0.15 μg/mL, 0.79 ± 0.07 μg/mL and 3.88 ± 0.17 μg/mL, respectively. Significant difference with respect to the background (saline-treated serum): $^{*}p < 0.05$.

were devoid of all carbonyl signals. Also, we previously demonstrated that the presence of trace volatile contaminant molecules such as formaldehyde and acetaldehyde, at concentrations up to 6.5 mM and 4.5 mM, respectively, do not activate complement in human serum (Moghimi et al., 2004). The levels of such volatile contaminants were negligible in current samples.

3.3. C4-dependent complement activation

C4d is a fluid phase degradation product of C4 cleavage, mediated by complement control protein C4bp and factor I, and an established marker of classical and lectin pathway activation (Fujita et al., 1978; Scharfstein et al., 1978; Hamad et al., 2008). Accordingly, further indication for the involvement of C4-dependent pathway was obtained by showing PEG-mediated rises of C4d in both normal (Fig. 3) and C2-depleted human serum (Fig. 2e). Low M_{wt} PEG species (e.g., PEG_{1960}) and PEG_{4240} (at concentrations below 10 mM), therefore, seem to activate complement exclusively via C4-dependent pathway, whereas PEG_{4240} (at 10 mM) and higher M_{wt} PEG species trigger complement through both C4-dependent pathway and accelerated alternative pathway turnover.

Next, we examined through which pathway(s) PEGs could initiate C4 cleavage. Complement activation through the classical pathway may be initiated by binding of naturally occurring antibodies against PEGs. These antibodies are of both IgG (predominantly IgG2 subclass) and IgM classes and their epitope is 4–5 repeat ethoxy units (—C—O—C—) irrespective of the end group moiety (Richter and Akerblom, 1984; Armstrong et al., 2007). Since Ca^{2+} is essential for the operation of the classical pathway (Szebeni et al., 1994, 1998; Moghimi et al., 2004, 2006), first we measured PEG_{1960}-mediated rises of both C4d and SC5b-9 in sera of five healthy individuals by excluding Ca^{2+} from the assay (PEG_{1960}, 10 mM, was used since it activated complement only through C4-dependent pathway). Remarkably, in all EGTA/Mg^{2+} supplemented sera, PEG_{1960} treatment significantly elevated both C4d and SC5b-9 levels above background (Fig. 4a), thus eliminating the role of classical pathway (no Bb elevation was detected in all tested sera, not shown). With longer PEG chains, C4d levels in EGTA-chelated sera were also elevated (not shown). As a control, Ca^{2+} chelation in serum halted cholesterol-rich liposome-mediated rises of C4d but not SC5b-9 level (Fig. 4b), as these vesicles activate complement through both antibody-mediated classical pathway as well as alternative pathway (Szebeni et al., 1994, 1997). The absence of anti-PEG antibodies in the five tested sera was confirmed next by an agglutination assay using the respective PEGylated autologous red blood cells (Armstrong et al., 2007). On the basis of these observations we also suggest that PEG-mediated triggering of the alternative pathway turnover is independent of anti-PEG antibody binding as antibodies can stimulate alternative pathway activation via their F(ab) portion (Moore et al., 1982). Despite these observations, still we cannot fully disregard PEG-mediated complement activation through the classical pathway in a serum with a high titer of anti-PEG antibodies, notably of IgM class (however, no such sera was available to us). To further eliminate the role of classical pathway, we examined PEG-mediated C4d rises in a C1q-depleted

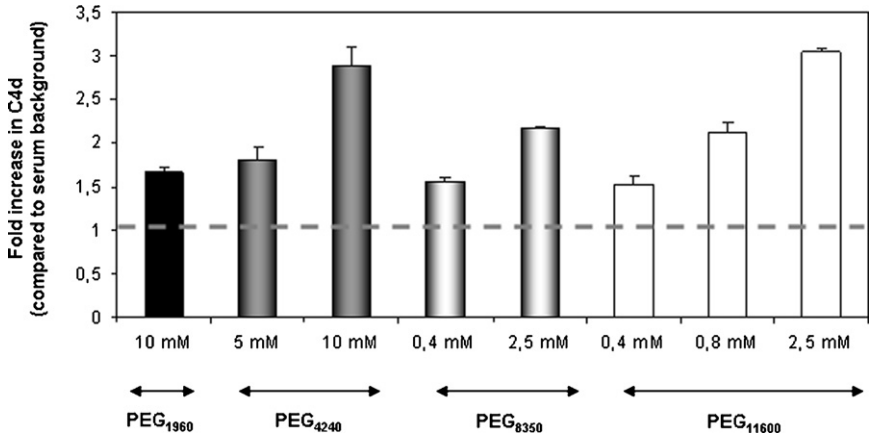

Fig. 3. PEG-mediated activation of the C4-dependent pathway. Serum source was the same as in Fig. 1. Serum C4d background level was 2.24 ± 0.16 μg/mL. Zymosan elevated serum C4d level by 2.95-fold, over background. In all treatments PEG elevated serum C4d levels significantly over the background ($p < 0.05$).

serum and the results (Fig. 5a) corroborate our findings with EGTA-chelated sera. In addition, these results collectively exclude a direct role for C1q in PEG-mediated complement activation.

Exclusion of the C1q-dependent pathway in PEG-mediated complement activation raises the intriguing question as to whether PEGs are capable of triggering complement via the lectin pathway. The lectin pathway initiator complex consists of either mannan-binding lectin (MBL) or ficolin and three MBL-associated serine proteases 1–3 (MASP-1, -2, -3) and the smaller non-enzymatic component sMAP (Fujita, 2002; Wallis, 2002). MBL bind to monosaccharides such as mannose, fucose and *N*-acetylglucosamine with affinities typically in mM range, where the sugar-binding site is localized around one of two Ca^{2+} sites of the carbohydrate-recognition domain (CRD) (Lee et al., 1991; Weis et al., 1992; Jack et al., 2001; Wallis, 2002). Equitorial hydroxyl groups at the 3- and 4-OH positions of the sugar residue serve as coordination ligands for the Ca^{2+} (Weis et al., 1992), but additional coordination ligands are further provided by asparagine and glutamic acid residues in the CRD that form hydrogen bonds with the equatorial 3-OH and the 4-OH groups. Ficolins, on the other hand, express specificity only for sugars with *N*-acetylated groups (Fujita, 2002) as well as acetylated compounds, relatively independent of the structure of the acetylated molecule (Krarup et al., 2004).

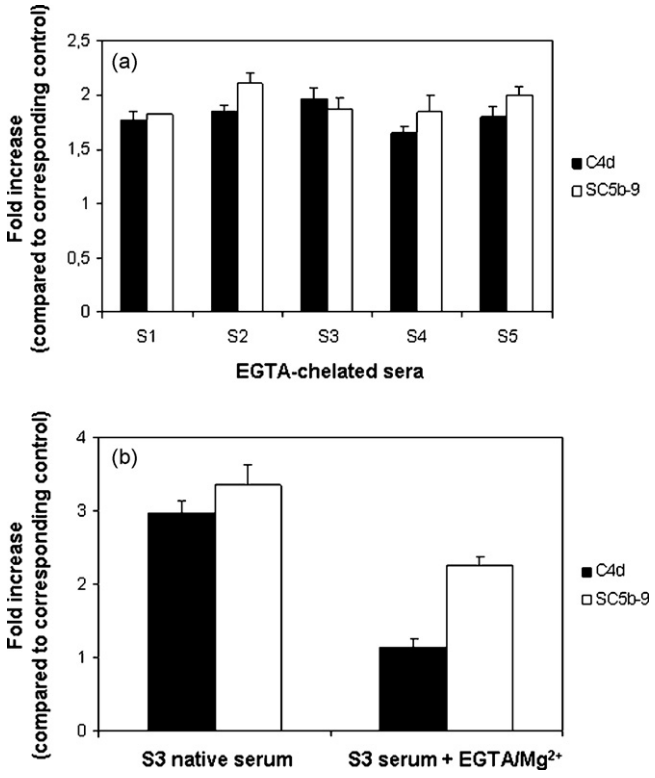

Fig. 4. Comparison of PEG_{1960}- and cholesterol-rich liposome-mediated complement activation in EGTA/Mg^{2+}-treated sera. Sera from five healthy male subjects with fully functional complement system were used (designated as S1–S5). PEG (10 mM)-mediated elevation of complement activation products is shown in panel (a), whereas panel (b) represents the effect of liposomes (3 mg lipid/mL) on serum SC5b-9 and C4d levels of subject 3 (S3) in the absence and presence of EGTA/Mg^{2+}. In all cases $p < 0.05$ with the exception of liposome-mediated C4d level in S3 + EGTA/Mg^{2+} (not significant) when compared with corresponding background levels of complement activation products.

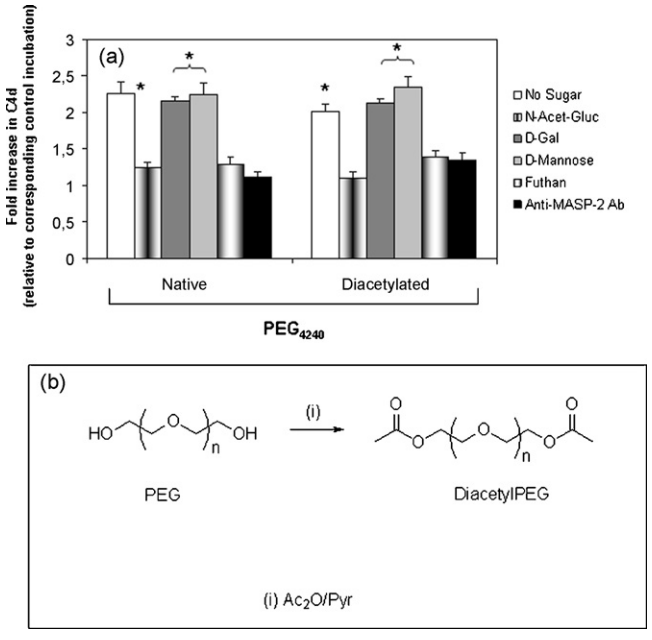

Fig. 5. The effect of native and diacetylated PEG_{4240} (10 mM) on C4d generation in C1q-depleted human serum. The C4d background level was 3.64 ± 0.37 μg/mL. PEG-mediated C4d levels were also measured by prior treatment of serum with sugars (25 mM final concentration), Futhan (150 μg/mL final concentration) and antibodies (anti-MASP-2 antibody and an irrelevant antibody). Sugar, Futhan, and irrelevant antibody addition had no significant effect on C4d background levels. Significant difference to the respective background: $^*p < 0.05$. Panel (b) represents the chemical structure of acetylated PEG. For the reaction chemistry refer to Section 2.

To address the above question, first we eliminated the possible involvement of MBL in PEG-mediated complement activation, since PEG treatment of C1q-depleted serum in the presence of 25 mM D-mannose elevated C4d levels but not when mannose was replaced with *N*-acetylglucosamine (Fig. 5a). D-Galactose was used as a non-antagonist (negative control). Interestingly, PEG-mediated C4d elevation in C1q-depleted serum was inhibited by both Futhan (a broad-spectrum serine protease inhibitor) (Pfeifer et al., 1999) and anti-MASP-2 antibodies (Hamad et al., 2008). This sensitivity to both *N*-acetylglucosamine and anti-MASP-2 antibodies strongly indicate a role for ficolins/MASP-2 in PEG-mediated complement activation and C4 cleavage and independent of calcium (Fig. 5a). These observations further raise the question as how PEGs interact with ficolins. When the OH groups at PEG termini was converted to acetyl groups (an established ligand for ficolin) (Fig. 5b), the complement activating ability (through C4d measurements) of resulting diacetylated PEGs remained practically similar to that of the native molecule and sensitive to Futhan, anti-MASP-2 antibodies and *N*-acetylglucosamine (Fig. 5a). This observation does not exclude the possibility that the OH groups can bind ficolin as efficiently as the acetyl groups. Despite these collective observations, PEG-mediated complement activation may still proceed through a MASP-2-dependent unconventional path and therefore requires further resolution.

4. Conclusions

We have demonstrated that PEGs can trigger complement activation by enhancing fluid phase complement turnover and a MASP-2-regulated process in a concentration- and M_{wt}-dependent manner. Our results are relevant to potential therapeutic applications of intravenous PEG where infusion doses are rather high, as in spinal cord injury and traumatic axonal brain injury, and warn about possible acute PEG infusion-related reactions. Similar to binding of allergens to IgE on the surface of mast cells and basophils, complement anaphylatoxins can trigger immediate release of various proinflammatory mediators (prostaglandins, leukotrienes, etc.) from these cells as well as macrophages in contact with the blood (Szebeni, 2005). This cascade of secondary mediators substantially amplifies effector immune responses and may induce anaphylaxis in sensitive individuals. Indeed, recent studies in pigs have demonstrated that systemic complement activation (e.g., induced following intravenous injection of PEGylated liposomes) can underlie cardiac anaphylaxis where C5a played a causal role (Szebeni et al., 2006). Cardiac mast cells express high-affinity receptors for complement anaphylatoxins and their triggering induces the release of a variety of inflammatory mediators and vasoactive molecules (Marone et al., 1995). C5a was also shown to intensify the allergen-induced anaphylactic crises in isolated, perfused guinea pig hearts (Ito et al., 1993). Complement activation, and sensitivity to anaphylatoxins and other related mediators, may therefore provide a plausible explanation to the previously reported unexplained anaphylaxis or the referred cardiovascular collapse in species that have received medicines containing high levels of PEG as solubilizer/carrier. In light of our observations, future experiments in relevant animal models and with anti-C5a antibodies are necessary to establish whether PEG-mediated peripheral complement activation can explain cardiac anaphylaxis. Finally, as a spin-off, near mono-dispersed PEGs may provide an additional platform for elucidation of ligand topology/structure for the pattern recognition molecules L- and H-ficolins, and subsequently illuminate the role of ficolins in defense as well as in endogenous homeostatic mechanisms.

Conflicts of interest

No conflicts of interest declared.

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