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Causative factors behind poloxamer 188 (Pluronic F68, Flocor™)-induced complement activation in human sera. A protective role against poloxamer-mediated complement activation by elevated serum lipoprotein levels

S. Moein Moghimi[a,*], A. Christy Hunter[a], Christopher M. Dadswell[b], Sandor Savay[c], Carl R. Alving[c], Janos Szebeni[c]

[a] *Molecular Targeting and Polymer Toxicology Group, School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewis Road, Brighton BN2 4GJ, UK*

[b] *School of Life Sciences, University of Sussex, Brighton BN1 9QJ, UK*

[c] *Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Silver Spring, MD 20910-3780, USA*

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Abstract

Poloxamer 188 is a complex polydisperse mixture of non-ionic macromolecules. Adverse non-IgE-mediated hypersensitivity reactions occur in some individuals following intravenous injection of poloxamer 188-based pharmaceuticals, presumably via complement activation. Here we have delineated potential causal chemical and biological interactive factors behind poloxamer 188-induced complement activation in human serum specimens. We identified the molecular constituents inherent in poloxamer 188 preparations and studied their effect on generation of the two complement split products, SC5b-9 and Bb. Poloxamer 188 activated complement at sub-micellar concentrations and the results indicated the potential involvement of all three known complement activation pathways. The poloxamer-induced rise of SC5b-9 in human sera was abolished in the presence of a recombinant truncated soluble form of complement receptor type 1, thus confirming the role of C3/C5 convertases in the activation process. Poloxamer 188-mediated complement activation is an intrinsic property of these macromolecules and was independent of the degree of sample polydispersity, as opposed to other non-polymeric constituents. Poloxamer 188 preparations also contained unsaturated chains of diblock copolymers capable of generating SC5b-9 in human sera; this effect was terminated following the removal of double bonds by catalytic hydrogenation. By quasi-elastic light scattering, we established interaction between poloxamer and lipoproteins; interestingly, poloxamer-induced rise in SC5b-9 was significantly suppressed when serum HDL and LDL cholesterol levels were increased above normal to mimic two relevant clinical situations. This observation was consistent with previously reported data from patients with abnormal or elevated lipid profiles where no or poor complement activation by poloxamer 188 occurred. Our findings could provide the basis of novel approaches to the prevention of poloxamer-mediated complement activation.

Keywords: Complement activation; Non-ionic surfactant; Poloxamer; HDL; LDL; Non-IgE-mediated hypersensitivity

1. Introduction

Poloxamer 188 (Pluronic F-68, Flocor™) in its entirety is a complex polydisperse mixture of non-ionic macromolecules [1]. Each molecule consists of a central polypropylene oxide chain flanked at either end by a polyethylene oxide segment, thus forming an ABA block copolymer structure. This copolymer has been widely tested in numerous experimental and clinical situations [2–7]. For example, intravenous injection or infusion of poloxamer 188 has been shown to be of significant benefit in the management of sickle cell

Abbreviations: SC5b-9, S-protein-bound form of the terminal complex; sCR1, recombinant soluble complement receptor type 1; PEG, poly(ethylene glycol); PEO, polyethylene oxide; cmc, critical micelle concentration; HSGC, head space gas chromatography; ECD, electron capture detector; ICP-AES, inductively coupled plasma atomic emission spectrometry; PBS, phosphate-buffered saline; CrEL, cremophor EL; MBL, mannose-binding lectin

* Corresponding author: Tel.: +44-1273-642-063; fax: +44-1273-679-333.

E-mail address: s.m.moghimi@brighton.ac.uk (S.M. Moghimi).

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disease as well as in stroke and myocardial infarction, in which poloxamer accelerates thrombolysis, reduces re-occlusion and ameliorates re-perfusion injury. Currently, this polymer is being evaluated for spinal cord injury and muscular dystrophy [8]. However, adverse non-IgE-mediated hypersensitivity reactions are known to occur in some individuals following intravenous administration of poloxamer 188-based pharmaceuticals. Such reactions were initially reported after the administration of perfluorochemicals emulsified with poloxamer 188 (e.g., Fluosol-DA) [9–15]. For example, Fluosol-DA infusion into rabbits and dogs has been associated with a transient, profound hemodynamic collapse accompanied by leukopenia and thrombocytopenia [9,10]. In a clinical study, up to 30% of patients receiving Fluosol-DA as an adjunct to radiation treatment of advanced squamous tumors of the head and neck developed hypotension and/or acute respiratory distress [13]. These adverse responses are believed to be secondary to complement activation by poloxamer micelles [10]. Recently, these reactions have been classified as ''complement activation-related pseudoallergy'' and also occur following infusion of contrast media, various drug solubilizers, and drug carriers [16–19], and presumably is a reflection of an individual's immune cell sensitivity to complement-derived mediators. In general, the incidence of pseudoallergic responses was recently estimated to be as high as 77% of all immune-mediated hypersensitivity reactions [17]. This estimate of the frequency rate of pseudoallergy would predict the occurrence of 420,000 severe pseudoallergic reactions with approximately 20,000 fatalities every year in the United States only [17]. Nevertheless, in view of the above applications for poloxamer 188 and the clinical importance of the observed complement-mediated hypersensitivity reactions, it is imperative to examine in detail and delineate the causal chemical and biological interactive factors behind poloxamer-induced complement activation in human sera. Understanding the molecular basis of these events may provide a platform for the design of new strategies to prevent poloxamer-mediated complement activation.

Macromolecules of similar structure but different molecular weight are known to initiate different pharmacological as well as immune responses [20,21]. Therefore, we have determined and assessed the molecular weight distribution and polydispersity of poloxamer preparations and studied their effect on generation of the two complement split products, the S-protein-bound form of the terminal complex (SC5b-9) and Bb, in human sera by enzyme-linked immunosorbent assay. This is a sensitive measure of the activation of the whole complement cascade that involves the formation of C3a and C5a anaphylatoxins [18,22]. Factor Bb, a soluble by-product of factor B, is also a specific marker of complement activation through the alternative pathway [18]. The role of C3/C5 convertases in poloxamer-mediated complement activation is also examined. In addition to polydispersity, the molecular make-up of poloxamer preparations consist of derivatives arising from the manufacturing process as well as storage conditions [1]. These constituents, which may show batch to batch variation, could be the underlying basis of complement-mediated adverse reactions. Hence, in this work, we have identified and quantified these molecular constituents, which consist of a range of low molecular weight volatiles, unsaturated diblock chains, and antioxidants, and studied their effect on generation of SC5b-9 in human sera. A low incidence of complement activation and complement-mediated adverse events have been reported following intravenous infusion of Fluosol-DA in to subjects with abnormal or elevated lipid profiles [9,10,14]. On the basis of these observations, we have also assessed interactions between poloxamer and isolated human lipoproteins and examined whether elevation of serum concentrations of LDL and HDL cholesterol relevant to two clinical situations (as classified by the National Cholesterol Education Program Expert Panel)[1] can exert a protective role against poloxamer-mediated complement activation.

2. Materials and methods

2.1. Materials

The non-clinical grade poloxamer 188 was a kind gift of BASF Aktiengesellschaft (Ludwigshafen, Germany) and was used as received unless chemically modified. Flocor™ (Lot 379-15-3), the clinical grade of poloxamer 188, was a gift of CytRx Corporation (GA, USA) and was used as received. Human HDL and LDL were purchased from Calbiochem (San Diego, CA, USA). Recombinant soluble complement receptor type 1 (sCR1) was provided by Avant Immunotherapeutics Incorporation (Needham, MA, USA).

2.2. Molecular weight determination

The molecular weight of the non-clinical grade poloxamer 188 was determined by the method of raised gel-permeation chromatography as described earlier [23]. Briefly, poloxamer was dissolved in dimethylformamide (2% w/v) and filtered through a 0.2 μm polyamide membrane prior to the chromatography. Sample solutions were passed through two columns (30 cm in length) of Polymer Laboratory Mixed Gel, 10.0 μm (RAPRA Technology, UK) at a flow rate of 1.0 ml/min at 80 °C. The eluent was monitored by measuring the refractive index. The system was calibrated with poly(ethylene glycol) (PEG), and polyethylene oxide (PEO) standards and the

[1] Third Report of the National Cholesterol Education Program Expert Panel on Detection, evaluation, and treatment of high blood cholesterol in adults (2002), Adults Treatment Panel III, NIH Publication No. 02-5215.

results are therefore expressed as the PEG/PEO equivalent molecular masses. Due to the limited amount of Flocor™, we were unable to independently determine its molecular weight by gel-permeation method; we quote values provided by the supplier.

2.3. Critical micelle concentration (cmc)

The cmc of non-clinical (BASF) and the clinical preparation (Flocor™) of poloxamer 188 was determined by a standard dye solubilization method at 37 °C. Briefly, known amounts of poloxamer 188 from a stock solution were added to stoppered glass vials in triplicate containing 2.5 mg/ml of the water-insoluble dye Sudan IV. Samples were sonicated for 2 min and incubated overnight at 37 °C in a shaking water bath. Following incubation, samples were filtered through a surfactant-free cellulose acetate 0.45 μm filter and the absorbance of each solution was measured spectrophotometrically at $\lambda = 515$ nm.

2.4. Hydrogenation of poloxamer 188

Poloxamer 188 (2 g) was reacted with a suspension of palladium on activated carbon (2 g, 10%) in ethyl acetate (100 ml), stirred under a hydrogen atmosphere for a period of 5 h at which point the reaction was complete. The catalyst was filtered out over a pad of celite and washed with ethyl acetate (500 ml), which was evaporated in vacuo to obtain the hydrogenation product. A control reaction was performed under identical conditions where hydrogen was replaced by nitrogen gas. Infrared spectra were measured directly by ATR using a Nicolet Avalar 320 FT-IR spectrometer and compared to the original poloxamer sample. ^{1}H-NMR spectra were determined in deuteriochloroform, with tetramethylsilane as an internal standard with a Bruker WM 360 spectrometer. All melting points were determined on an Electrothermal melting point apparatus.

2.5. Head space gas chromatography (HSGC)

A Perkin-Elmer Autosystem XL with an electron capture detector (ECD) instrument was used. The column type was an SGE BPX5, 10 m × 0.1 mm ID, 0.1 μm film thickness. The oven program was set at 50 °C (1 min), then 5 °C/min to 110 °C, then 20 °C/min to 220 °C, and finally held at 220 °C for 2 min. The carrier gas used was helium at 45 psi with an injector temperature of 200 °C and an ECD temperature of 375 °C. Injector mode was set at split. Poloxamer 188, the hydrogenated derivative or Flocor™ (10 mg) was placed into a 2 ml gas chromatography vial to which 480 μl of HPLC grade water was added (final volume 500 μl). An internal standard 1,2-dibromopropane (0.2 ppm) was then introduced into the vial (10 μl of a 10 ng/μl solution of 1,2-dibromopropane in methanol). For standard addition analysis, a volume of the polymer solution (100 ng/μl of each of the target compounds in HPLC grade water) was added. Analysis was performed using this solution at a range of volumes of between 2.5 and 10 μl. To this, 500 μl of the derivatization solution was added [1 mg/ml *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride in HPLC grade water]. Derivatization enabled us to use ECD as a detector to increase sensitivity by a few orders of magnitude. In addition, derivatization circumvented matrix interference, which can result in false low readings. The gas chromatography vial was then closed with a PTFE-lined septum cap and mixed thoroughly then placed into an oven at 60 °C for 15 min. A volume for headspace dispersion (typically 50 μl) was introduced into the gas chromatography injection port using a pre-heated 250 μl gas tight syringe for analysis.

2.6. Inductively coupled plasma atomic emission spectrometry (ICP-AES)

This technique was used to determine the concentration of Mg^{2+} and Ca^{2+} ions in the various poloxamer samples. Initially calibration curves were constructed with standard solutions of Mg^{2+} and Ca^{2+} over a concentration range of 0–10 ppm and measured at emission wavelengths of $\lambda = 279.553$ and $\lambda = 393.366$, respectively, using a Perkin-Elmer Plasma 400. Samples at a concentration of 5 mg/ml in HPLC grade water were analysed at a flow rate of 1 ml/min.

2.7. Subjects and collection of serum specimens

Blood was drawn from healthy volunteers according to protocols approved by the Walter Reed Army Medical Center Human Use Committee. Blood was allowed to clot at room temperature and serum was prepared, then stored at − 80 °C. Serum samples were thawed and kept at 4 °C before incubation with test agents.

2.8. Assay of in vitro complement activation

To measure complement activation in vitro, we determined the poloxamer 188-induced rise of the two complement split products, SC5b-9 and Bb, using enzyme-linked immunosorbent assay kits (Quidel Co., San Diego, CA, USA) as described in detail elsewhere [18,19]. Briefly, poloxamer 188 or the modified forms of the copolymer were incubated (at different concentrations) with undiluted human serum for 30 min at 37 °C in a shaking water bath (in triplicate determinations), at an activator to serum volume ratio of 1:4. Typically, 10 μl of the poloxamer stock solutions were added to 40 μl of serum placed in Eppendorf tubes. In some experiments, the additive effects of human HDL, LDL and organic solvents on poloxamer 188-induced rise of the two complement split products were measured. Zymosan was used as a control to ensure the presence of functional complement system in human sera. In all experi-

ments, 10 mM phosphate buffered saline (PBS), pH 7.4, was used as control.

2.9. Size analysis of human HDL and LDL

The hydrodynamic size of human lipoproteins were measured both in the absence and presence of Flocor™ (5 mg/ml final concentration) by quasi-elastic light scattering, using a Nicomp Model 370 (Pacific Scientific, Silver Spring, MD, USA). All measurements were done in 10 mM PBS, pH 7.4, at 24 °C as described in detail elsewhere [19]. Size measurements (volume weighting mode) were determined at a wavelength of 633 nm, scattering angle of 90°, dispersant viscosity of 0.90 cP and refractive index of 1.33, respectively.

2.10. Statistical analysis

Data from the in vitro complement assays are expressed as mean of triplicate determinations ± S.D. Since various tests (poloxamer 188 and its derivatives, contaminants, modulators) and control groups (negative control PBS, and positive control zymosan) were run at the same time, we had ≥ four groups. These were subjected to one-way ANOVA followed by the Student–Neuman–Keuls post hoc test, as indicated by the respective experiments. The analysis of complement activation in 17 sera was performed by ANOVA followed by the Bonferroni post hoc test, comparing only the baseline and test samples for each serum. Differences between groups were considered significant at $P < 0.05$.

3. Results

3.1. Analysis of poloxamer 188 samples

Gel-permeation chromatography has demonstrated that the non-clinical poloxamer grade is polydisperse and is composed of molecules that range from a molecular weight < 4500 Da (3.4% of total) to over 13,000 Da (6.8% of total), with a weight average molecular weight of 7361 Da (Table 1). In contrast, the clinical preparation of poloxamer 188 (Flocor™) has a narrow polydispersity and weight average molecular weight of 8566 Da (analysis data was provided by the supplier). Flocor™ contained 1.1% of low (< 4500 Da) and 1.0% of high (>13,000 Da) molecular weight components. The non-clinical poloxamer preparation was unsaturated to the extent of 0.02 mEq/g as determined by titration with mercuric acetate. The unsaturated molecules separated overwhelmingly into the lower molecular weight fraction during gel-permeation chromatography. Removal of double bonds in the whole mixture was achieved by atmospheric pressure hydrogenation and confirmed by spectroscopy where IR absorption signal corresponding to double bonds (1650 cm^{-1}) present in the starting material and the control was absent in the hydrogenation product. Comparison of the ^{1}H-NMR spectra of the starting material and hydrogenation product confirmed the catalytic hydrogenation did not lead to any degradation in the hydrogenated product. No unsaturated chains were detected in Flocor™. Both non-clinical and clinical preparations of poloxamer contained organic volatiles, particularly acetaldehyde and propionaldehyde, as determined by HSGC-ECD (Table 2). However, the quantity of organic volatiles was considerably lower in the hydrogenated preparation of poloxamer 188 and Flocor™.

In aqueous solution, poloxamer 188 forms micelles and these structures are believed to initiate complement activation. Therefore, we assessed the cmc of poloxamer 188 as well as micellar size distribution. The micelle formation in poloxamer 188 solutions was temperature sensitive. In accordance with previous studies [24], we were unable to establish a cmc for poloxamer 188 at room temperature (25 °C) by established techniques. However, at physiological temperature, both non-clinical and clinical preparations of poloxamer 188 formed spherical micelles with a cmc in the range of 24–32 and 29–38 mg/ml, respectively, and are in agreement with previous reported values [24]. The average hydrodynamic diameter for both types of micelles was < 20 nm at 37 °C as determined by photon correlation spectroscopy (Table 1).

Table 1
Properties of poloxamer 188 and Flocor™

Property	Poloxamer 188	Flocor™
Weight average MW (Da)[a]	7361	8566
Peak Mw (Da)[a]	8923	8753
Polydispersity[a]	1.29	1.04
Low Mw components (% of total)[a]	3.4%	1.1%
High Mw components (% of total)[a]	6.8%	1.0%
CMC at 37 °C (mg/ml)	24–32	29–38
Z-average mean micelle size	14.6 ± 3.1 nm	12.5 ± 2.2 nm
Unsaturation (mEq/g)	0.02	not detected
Mg^{2+} content (μg/l)[b]	7.0	not detected
Ca^{2+} content (μg/l)[b]	9.0	30.0
Melting point range	51–53 °C	51–52 °C

[a] Values for Flocor were taken from its certificate of analysis (CytRx Lot 379-15-3).

[b] Determined from a solution containing 5.0 g/l of either poloxamer 188 or Flocor™.

Table 2
HSGC-ECD determination of volatile degradation products of interest in poloxamer 188 and Flocor™ samples

Test sample	Measured products (μg/g)			
	Formaldehyde	Acetaldehyde	Acetone[a]	Propionaldehyde
Poloxamer 188	0.7	188.0	not detected	145.6
Hydrogenated Poloxamer 188	3.4	10.8	not detected	5.2
Flocor™	1.2	55.9	not detected	21.7

[a] Estimated detection limit for acetone was 1.0–5.0 μg/g.

3.2. Complement activation by poloxamer and the role of convertases

Complement activation was assessed by measuring the production of SC5b-9 in human sera. The data in Fig. 1 shows the changes of SC5b-9 level in sera of 17 healthy individuals after 30 min incubation with the non-clinical preparation of poloxamer 188 below its cmc (5 mg/ml final concentration) at 37 °C. These observations established statistically significant rises of serum SC5b-9 over the control incubation (PBS) in 13 of 17 individuals. The complement response to poloxamer varied considerably in the individual sera, with rises in SC5b-9 levels up to 3-fold. The results identified four non-responders (G, H, I, N) where poloxamer failed to initiate complement activation although these sera contained functional complement proteins, as complement activation did proceed following challenge with zymosan resulting in generation of high levels of SC5b-9 (data not shown).

The results in Fig. 2a demonstrate the effect of poloxamer 188 concentration on complement activation in a typical responder serum. A poloxamer concentration as low as 2.5 mg/ml was found to initiate complement activation. Increasing the concentration of poloxamer increases the production of SC5b-9 in human serum. Therefore, at concentrations below its cmc poloxamer 188 monomers can activate the human complement system. Attempts were also made to determine the pathway of complement activation by the poloxamer. By excluding Ca^{2+} from the assay, alternative pathway activation can be distinguished easily from the classical and mannose-binding lectin (MBL) pathways, as Ca^{2+} is essential to the operation of the later pathways [18].

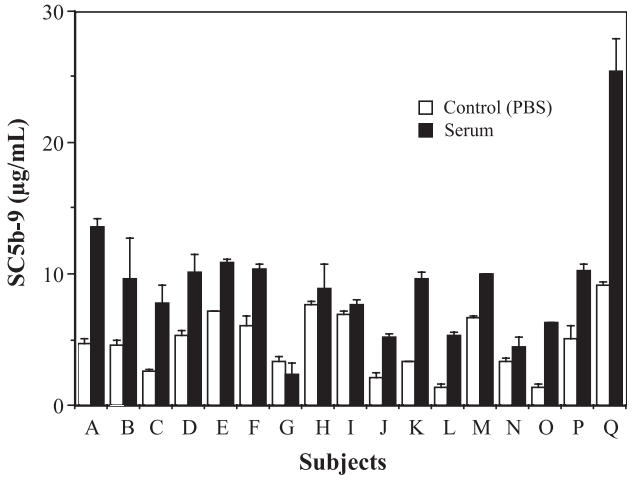


Fig. 1. Effect of poloxamer 188 on SC5b-9 formation in different sera obtained from healthy subjects. Poloxamer 188 (5 mg/ml) or a corresponding volume of PBS, as control, was incubated with serum samples from 17 healthy volunteers and the level of SC5b-9 was determined. The difference between poloxamer 188 and PBS was statistically significant for all specimens ($P<0.001$ for A, B, C, D, K, O, P and Q; $P<0.01$ for E, F and L; $P<0.05$ for J) except G, H, I and N ($P>0.05$).

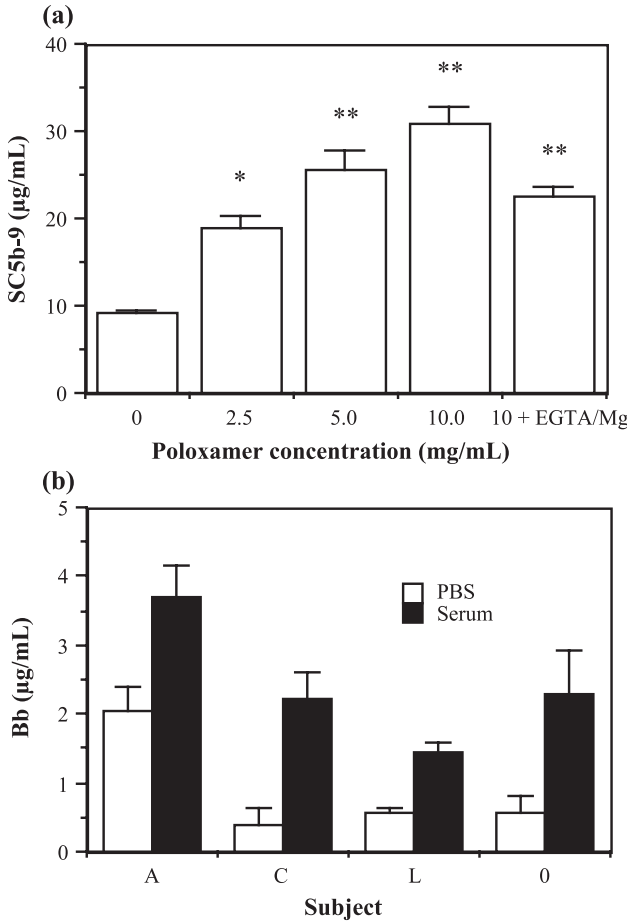


Fig. 2. Effect of poloxamer on SC5b-9 and Bb formation. In (a) the effect of poloxamer concentration on SC5b-9 formation in a representative human serum is demonstrated. Similar patterns were observed with all other tested sera; $*P<0.05$ and $*P<0.01$ compared to control (0 mg/ml). The addition of EGTA/Mg^{2+} caused partial inhibition of Sc5b-9 formation. The difference between poloxamer incubation (10 mg/ml) and poloxamer (10 mg/ml) in the presence of EGTA/Mg^{2+} was statistically significant ($P<0.05$). In (b) activation of complement via the alternative pathway is further confirmed by an increase in serum levels of Bb from four healthy subjects. The difference between poloxamer (5 mg/ml) and control (PBS) was statistically significant in each subject ($P<0.01$).

The results in Fig. 2a shows that poloxamer can still cause the formation of the SC5b-9 complex in Ca^{2+}-depleted serum, but the concentration of SC5b-9 is significantly lower ($P<0.01$) in EGTA-chelated serum. These results provide evidence that although poloxamer-induced complement activation is predominantly via the alternative pathway, a role for the classical and the MBL pathways cannot be excluded. Since operation of the alternative pathway is maintained or even accelerated by elevated Mg^{2+} levels, we monitored the content of both Ca^{2+} and Mg^{2+} in poloxamer samples by ICP-AES. The results confirmed negligible quantities of these cations in all samples (Table 1) and hence complement activation via the alternative pathway is not driven by sample contamination with Mg^{2+}. To examine individual variation in complement activation via the alter-

native pathway, poloxamer-mediated rise in serum Bb levels was further monitored in the sera of responders. In all tested sera, the poloxamer-induced rise of SC5b-9 was indeed associated with a substantial individual variation in the extent of serum Bb rise; some examples are given in Fig. 2b. The poloxamer-induced rise of SC5b-9 in all normal sera was totally inhibited in the presence of 10 μg/ml of a recombinant truncated soluble form of complement receptor type I (sCR1), two typical examples are given in Fig. 3. This molecule inhibits complement activation both by accelerating the decay of C3 and C5 convertases, and by acting as a co-factor for the proteolysis of C3b and C4b by factor I [25,26].

3.3. The role of unsaturation and sample polydispersity in complement activation

We evaluated whether the presence of unsaturated diblock copolymers is a causal factor contributing to activation of the complement cascade. This possibility was tested in the serum of responders using the hydrogenated poloxamer 188. In order to ensure that a contaminant was not responsible for the complement activation by the hydrogenated product, a control poloxamer 188 sample was prepared where hydrogen was replaced by nitrogen but otherwise under identical conditions. Two different patterns were observed. In some sera the hydrogenated product also caused complement activation (a representative example is subject 1), whereas in others (as represented by subject 2) poloxamer-induced rise of SC5b-9 was abolished by prior poloxamer hydrogenation (Table 3). Since unsaturated molecules also separate overwhelmingly into the lower molecular weight fraction during gel-permeation chromatography, complement activation was also assessed with the peak fraction (a fraction devoid of unsaturated molecules as well as high molecular weight components). Again, the results were comparable with those of the hydrogenated product in all serum specimens (data not shown). To further determine the role of unsaturation as well as polydispersity in complement activation, Flocor™ was used. This attempt demonstrated statistically significant rises of serum SC5b-9 in all responders; two representative cases are presented in Table 4. This provides clear evidence that Flocor™, although exhibiting low polydispersity and no unsaturation, can cause complement activation. Again, Flocor™ failed to raise SC5b-9 levels in the serum of subject 2 (as an example of a case that did not respond to hydrogenated poloxamer 188) and the values were comparable with the control incubation (data not shown).

Table 3
Comparison of complement activation between poloxamer 188 and its hydrogenated product in sera of two healthy subjects

Incubations	SC5b-9 (μg/ml)	
	Subject 1	Subject 2
Control (PBS)	6.1 ± 0.8	3.5 ± 0.2
Zymosan	27.4 ± 2.5***	11.3 ± 0.6***
Poloxamer 188	10.1 ± 1.6*	5.1 ± 0.3*
Hydrogenated poloxamer	14.9 ± 1.6**	2.1 ± 0.1

All p values are compared with control (PBS) and arise from one-way ANOVA followed by the Student–Neuman–Keuls post hoc test.
*$p<0.05$.
**$p<0.01$.
***$p<0.001$.

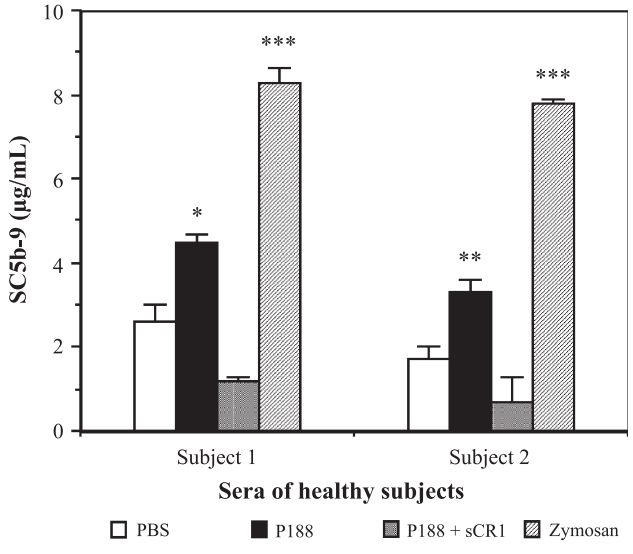


Fig. 3. Inhibition of poloxamer-induced complement activation by sCR1. Inhibition occurred in all tested sera and two representative cases are presented. The rise of SC5b-9 relative to the respected control (PBS) was statistically significant: *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

3.4. The role of residual organic solvents and antioxidants in complement activation

Experiments were also performed to assess whether complement activation by poloxamer samples is due to the presence of those residual organic solvents that are detected by GC-ECD. None of the volatile molecules (final concentration 0.2 mg/ml) either separately or in combination caused elevation of serum SC5b-9 and in all cases (four sera that responded to both poloxamer 188 and Flocor™ were tested) the values were comparable to the control

Table 4
Additive effect of HDL and LDL on Flocor™-mediated complement activation in sera of two representative healthy individuals

Treatment	SC5b-9 (μg/ml)	
	Subject 3	Subject 4
Control (PBS)	2.7 ± 0.2	4.1 ± 0.7
Zymosan	12.0 ± 1.9***	23.9 ± 1.3***
Flocor™	7.2 ± 0.8***	8.4 ± 0.2***
Flocor™ + HDL	5.4 ± 0.4†/**	5.1 ± 0.3$^{\dagger\dagger\dagger/\perp}$
Flocor™ + LDL	4.8 ± 0.4†/**	3.3 ± 0.2$^{\dagger\dagger\dagger/\perp}$

All p values arise from one-way ANOVA followed by the Student–Neuman–Keuls post hoc test, compared with control (PBS): **$p<0.01$, ***$p<0.001$, $^{\perp}p>0.05$ (not significant); compared with Flocor™: $^{\dagger}p<0.05$ and $^{\dagger\dagger\dagger}P<0.001$.

incubation (data not shown). Furthermore, the volatile molecules also failed to potentiate complement activation by Flocor™ in these sera. Other likely contaminants such as antioxidants (e.g., butylated hydroxytoluene) did not have a role in complement activation.

3.5. Interaction of human lipoproteins with poloxamer 188 and modulation of complement activation

We evaluated the possible interaction between human lipoproteins and Flocor™ and their effects on the complement cascade. Results in Fig. 4 show quasi-elastic light-scattering analysis of the hydrodynamic size of isolated human lipoprotein fractions both in the absence and presence of Flocor™ (5 mg/ml). We were unable to detect any particles in a 5 mg/ml solution of Flocor™ (low photon counts) thus confirming the absence of any micellar structures. The hydrodynamic size distribution of HDL and LDL are in agreement with their expected size (8–11 nm for HDL and 20–30 nm for LDL). In the presence of Flocor™, the hydrodynamic size of HDL is increased as evident from a shift to the right in the size-distribution profile. Interestingly, the addition of Flocor™ generates two populations of particles from LDL that differ in size distribution. One population is classified as small-sized entities (10–20 nm) with the majority displaying a hydrodynamic diameter of 15 nm, and a second population with particles in the range of 30–50 nm, where the majority is in the region of 28–32 nm. These observations support an interaction between Flocor™ and lipoproteins. These observations were reproducible with different lipoprotein preparations.

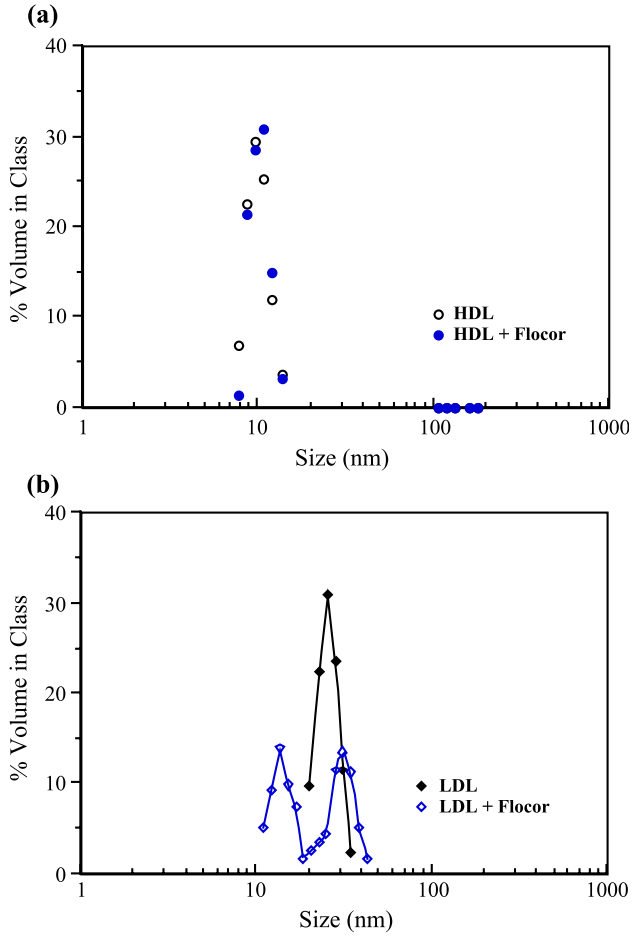


Fig. 4. The effect of Flocor™ (5 mg/ml) on hydrodynamic size distribution of isolated human serum (a) HDL and (b) LDL. Nicomp distribution analysis was performed in the volume-weighting mode plotting the log of particle size against relative volume. No particles were detected in a 5 mg/ml solution of Flocor™. The composition of HDL was 2.33 mg/ml protein, 0.61 mg/ml cholesterol and 0.20 mg/ml triglycerides. The composition of LDL was 0.94 mg/ml protein, 1.88 mg/ml cholesterol and 0.23 mg/ml triglycerides. The above pattern was reproducible in all other preparations tested.

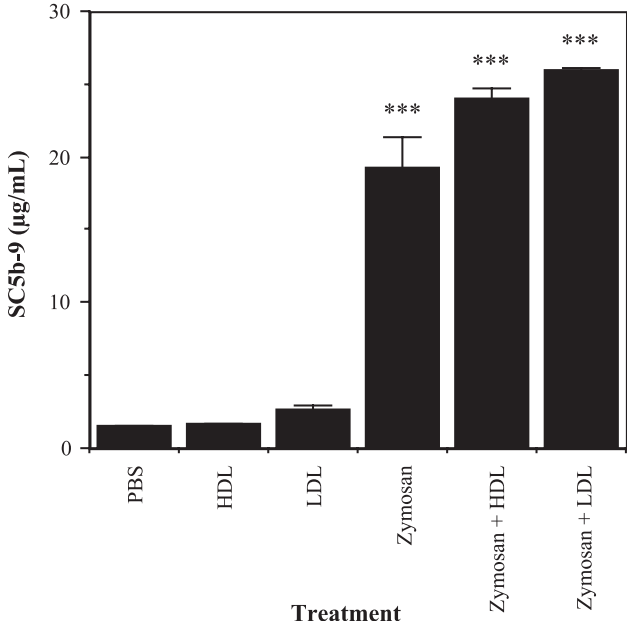


Fig. 5. Effect of HDL and LDL on SC5b-9 formation in serum of a healthy individual. The rise of SC5b-9 following HDL (HDL cholesterol added = 35 mg/dl) and LDL (LDL cholesterol added = 188 mg/dl) addition relative to control (PBS) was statistically not significant ($P > 0.05$). The difference between PBS and zymosan incubations were statistically significant, ***$P < 0.001$. The above pattern was reproducible with all sera tested.

The additive effect of lipoproteins on poloxamer-mediated complement activation in the sera of two representative responders with normal cholesterol (~ 180 mg/dl) and triglyceride (~ 200 mg/dl) levels is presented in Table 4. The addition of HDL to serum specimens increased HDL cholesterol levels by 30%. Such increased HDL cholesterol levels are considered beneficial and represent a target for many therapies.[1] The increased cholesterol levels following LDL addition mimicked the levels that are encountered in heterozygous familial hypercholesterolemia (LDL cholesterol added = 188 mg/dl), which occurs in 1/500. The results in Table 4 show that the elevated serum HDL and LDL cholesterol levels can suppress Flocor™-mediated SC5b-9 production. This suppression is more effective with LDL than HDL. Furthermore, suppression of complement acti-

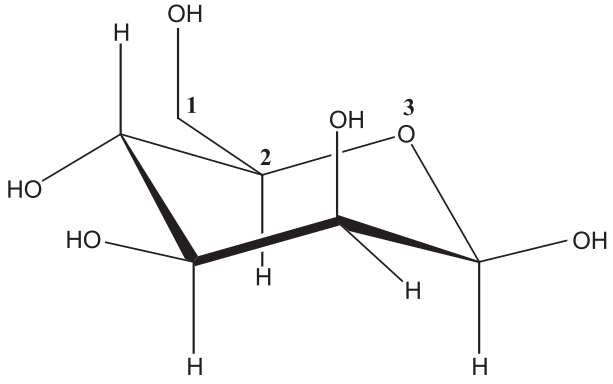
vation is not due to the presence of a complement inhibitor in lipoprotein preparations since zymosan-mediated complement activation did proceed in the presence of added lipoproteins; a representative example is presented in Fig. 5 and similar patterns were observed with other tested sera. In the absence of poloxamer, the elevated serum levels of HDL and LDL cholesterol had no significant effect on SC5b-9 production (Fig. 5).

4. Discussion

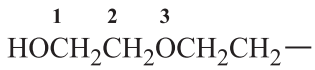
There has been a long debate as to whether complement activation in human serum by poloxamer 188 arises from its polydisperse nature (particularly by those populations with molecular weights of ≥ 12 kDa), the presence of other polymeric derivatives or non-polymeric constituents [10,20,27,28]. Therefore, in this work, we have identified and quantified the molecular constituents inherent to poloxamer 188 preparations and studied their effect on complement activation in human sera.

Our results with the non-clinical poloxamer grade as well as Flocor™, clearly demonstrate that poloxamer-mediated complement activation, as demonstrated with a dramatic rise in SC5b-9 levels in sera of healthy individuals, is irrespective of the degree of copolymer polydispersity. Furthermore, our observations with both Flocor™ and the peak poloxamer 188 fraction from gel-permeation chromatography (a fraction devoid of molecular weight components >10 kDa) do not support an exclusive role for the high molecular weight components as a causative factor in complement activation. Our attention was further focused on other constituents. Poloxamer 188 is formed by condensation of propylene oxide and ethylene oxide under an inert anhydrous atmosphere at elevated temperature and pressure in the presence of an alkaline catalyst (usually KOH) [29]. Under such conditions, elimination of a proton from either the propylene oxide or ethylene oxide reactants can generate an allylic double bond. Therefore, only one chain-end will be available to react with ethylene oxide in the second part of the poloxamer synthesis. As a result, chains of diblock copolymers are also formed that are allyl ether terminated on the propylene oxide side and hydroxyl terminated on the ethylene oxide side [30]. Indeed, this study has demonstrated, that in addition to poloxamer chains, allylic double bonds also play some role in complement activation, since their removal by hydrogenation can totally abolish this effect in some sera. There are also non-polymeric constituents in poloxamer samples [1,27,28,31]. For instance, thermal oxidation of poloxamer can occur during manufacturing, and is dominated by the initial formation of hydroperoxides, which form primarily at secondary carbons on the polymer backbone. The presence of oxygen will accelerate the reaction. The volatile products of thermal degradation, may include formaldehyde, acetaldehyde, acetone and propionaldehyde. These contaminants could increase C3 conversion in normal human serum particularly through inhibition of factor I [32]. However, our experimental work with both non-clinical and clinical preparations of poloxamer 188 does not support a primary role for volatile contaminants and antioxidants in the generation of SC5b-9. On the basis of the above observations, we strongly suggest that poloxamer-mediated complement activation in human sera is an intrinsic property of its polymeric constituents.

Poloxamer 188-induced complement activation appears to be a complex process, and exhibits substantial interindividual variations. Poloxamer-induced rise of serum SC5b-9 was sensitive to inhibition by EGTA, at least in part, and serum Bb levels also increased following poloxamer addition to serum. These observations indicate the potential involvement of all three known complement activation pathways. Complement activation through the classical pathway is presumably initiated by the binding of naturally occurring antibodies to poloxamer segments or to diblock copolymers containing the allylic double bonds. However, considering that complement activation by poloxamer occurred at sub-micellar concentration, this hypothesis hinges on the functional viability of antibody (mainly IgM)-poloxamer mono- and oligomeric complexes. Furthermore, calcium-dependent complement activation can also proceed through direct binding of C1q or MBL to the activator. Interestingly, there are structural similarities between the terminal PEO segments of the poloxamer and a region of D-



D-Mannose



Terminal region of poloxamer

Fig. 6. Structures of D-mannose and the terminal region of poloxamer 188. Structural similarities correspond to positions 1, 2 and 3. Only one terminal end of poloxamer is shown, the other is identical.

mannose (Fig. 6). Thus, poloxamer-mediated complement activation via the lectin pathway is also possible; a hypothesis that needs to be proved in future studies. The involvement of the alternative pathway in complement activation by poloxamer 188 was demonstrated by the increased serum Bb levels over control. This activation may represent amplification of C3 convertase initially triggered through the calcium-sensitive pathways, or increased ''C3-tick-over'', whereupon the highly hydrated PEO chains of poloxamer 188 initiates increased hydrolysis of the thioester bond in C3 to form C3(H_2O), which binds factor B and enables the bound factor B to be cleaved by factor D thus generating soluble alternative pathway C3 convertases. In our system, complement activation could also proceed via some unusual routes such as direct interaction with C3 and C4 and a conformational change, leading to products resembling activated C3 and C4 [16]. However, this possibility is unlikely since complement activation by poloxamer 188 was totally abolished in the presence of sCR1, an inhibitor of C3/C5 convertases. Finally, poloxamer could inhibit factors H and I, thereby enhancing alternative pathway activation and/or amplification. The observed individual differences in SC5b-9 and Bb responses to poloxamer are also similar to those reported previously with the non-ionic surfactant Cremophor EL (CrEL), the solubilizing agent for paclitaxel [19]. These differences can be attributed to the biological variation in the plasma levels of some 15 complement proteins and the large number of positive and negative feedback interactions.

Earlier work in human serum demonstrated that CrEL not only caused complement activation but also substantially decreased the electrophoretic mobility of both HDL and LDL [33,34]. The changes in electrophoretic mobility were associated with a shift of HDL and LDL species to a larger molecular size with no evidence of lipoprotein dissociation into small fragments [34]. The data suggested that these alterations were due to incorporation of some of the hydrophobic components of CrEL into lipoproteins [33]. Recently, it was suggested that the components of CrEL that did not associate with lipoproteins could form large lipid structures that assemble C3 convertases [19]. Thus, this structural transformation in serum appears to account for complement activation by CrEL. Our results with HDL and LDL also show that Flocor™ can interact with lipoproteins, and alter their size. Unlike CrEL, poloxamer molecules caused LDL dissociation and generated a population of small-sized particles. Furthermore, the addition of Flocor™ (up to 20 mg/ml) to serum did not result in the formation of unusual or large new structures that could act as a platform for the assembly of C3 convertases, as assessed by both electron microscopy and quasi-light scattering (data not shown). Remarkably, we found that the elevation of serum HDL and LDL cholesterol levels above normal (mimicking the relevant clinical situations) can significantly reduce poloxamer-mediated complement activation. This preliminary observation, however, is in line with low incidence of poloxamer-mediated complement activation in subjects with abnormal or elevated lipid profiles [9,10,14]. The hypothesis that the elevated lipoprotein levels could provide protection against complement activation by poloxamer 188 is worthy of exploration at a clinical level. The molecular basis of this interaction is not clear but may be mediated between the PEO chains of the poloxamer and apolipoproteins [35,36]. Indeed, poloxamer-coated polystyrene nanospheres become surface-enriched with apolipoproteins following incubation in human serum [35].

A side effect of complement activation is the generation of anaphylatoxins C3a and C5a [17]. These molecules can induce potent biological responses at very low concentrations and are involved in inflammatory and anaphylactic reactions. Circulating leukocytes, mast cells, macrophages, etc., express receptors for C3a and C5a. Activation of these cells by C3a and C5a results in the release of inflammatory mediators such as histamine and pro-inflammatory cytokines, which in turn alter vascular permeability, induce smooth muscle contraction and cause inflammatory cell migration [22,37]. If complement activation is a contributor to the hypersensitivity reactions to poloxamer or Flocor™, then sCR1 might provide prevention. Previous in vitro studies have shown sCR1 to be a potent inhibitor of complement-mediated harmful effects in 10–40 μg/ml concentration range [25,26,38]. We also demonstrated that sCR1 can effectively inhibit poloxamer-induced complement activation at 10 μg/ml.

In summary, for the first time we have shown that poloxamer 188-mediated complement activation is an intrinsic property of its macromolecular components and is irrespective of the degree of sample polydispersity. In addition, we hypothesised that plasma lipoproteins may control and modulate the extent of complement activation. In the majority of sera tested, poloxamer did cause complement activation and if complement-derived mediators are contributors to hypersensitivity, then our findings could provide the basis of novel approaches to the prevention of poloxamer-mediated complement activation either by new strategies in therapeutic copolymer design or by biochemical means. However, the ultimate goal is to understand the molecular mechanism of ''complement activation-related pseudoallergy'', which operates in a small population of individuals. Future developments in immunogenomics and predictive gene-derived toxicogenomic may eventually provide new methods for assessing an individual's sensitivity to polymeric therapeutics and hence reduce the risk of immunotoxicity.

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