Complement activation by pegylated liposomes containing prednisolone

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ABSTRACT

Infusion of PEGylated liposomes can give rise to hypersensitivity reactions (HSRs) in a relatively large number of patients. Previously it has been shown that these reactions can be caused by activation of the complement (C) system by a negative charge on the anchor molecule of PEG at the liposomal surface. In this study it is tested whether the activation of the C system by PEG-liposomes could be significantly reduced to values comparable to nonreactive liposomal formulations, by changing the PEGylation-profile on the liposomal surface. Therefore, the formation of C activation markers SC5b-9, C3a, C4d and Bb in normal human serum by both prednisolone loaded and empty liposomes with a variation of PEG chain length, PEG surface concentration, PEG anchor molecule and liposomal size was determined using in vitro assays. The tested liposomes caused no or only mild (30%) activation of C except for one formulation wherein the PEG2000 was anchored to cholesterol (CHOL-PEG2000). The latter liposomes caused paralleling rises in SC5b-9 and Bb levels, suggesting excess activation of the alternative pathway. While the relative safety of weak C activator liposomes remains to be confirmed in vivo, the unique, non-charge and non-antibody-mediated direct conversion of C3 by CHOL-PEG2000 liposomes—although argued against the clinical development of these vesicles—opens new opportunities to understand liposomal C activation at the molecular level.

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1. Introduction

Liposomes have been investigated extensively as drug delivery vehicles to increase the therapeutic index of the encapsulated drug, and their versatility to accommodate a wide range of therapeutic agents has been demonstrated in numerous preclinical and clinical settings (Metselaar et al., 2002). Surface modification with a hydrophilic polymer layer, such as poly-ethylene glycol (PEG), opposes their uptake by cells of the mononuclear phagocyte system (MPS) and therefore results in prolonged circulation times. Such long-circulating PEGylated liposomes have demonstrated passive targeting to tumor tissues and inflamed sites, as the locally enhanced vascular permeability allows these drug carriers to extravasate by virtue of the enhanced permeability and retention (EPR) effect. This is one of the mechanisms that improve the therapeutic index of the encapsulated drug (Duncan et al., 2005; Maeda et al., 2000; Reddy, 2000).

However, infusion of PEGylated liposomes can give rise to hypersensitivity reactions (HSRs) in a relatively large number of patients (numbers of up to 30% have been reported) (Chanan-Khan et al., 2003; Szebeni et al., 2002, 2012). The reaction usually occurs at the start of infusion and includes symptoms like cardiopulmonary distress, hypo- or hypertension, dyspnea, tachypnea, facial edema and pain in the chest and back. Most of these HSRs are transient and mild, but life-threatening reactions also have been documented in hypersensitive patients (Bates et al., 1995; Chanan-Khan et al., 2003; Laing et al., 1994; Moghimi et al., 2010; Schneider et al., 1998; Szebeni et al., 2012; Vaidya et al., 2002). Since these reactions occur at the first exposure to the drug (without prior sensitization), they are often referred to as ‘pseudoallergy’ and (because of the causal or contributing role of complement activation) the phenomenon is called ‘complement activation related pseudoallergy’ (CARPA) (Szebeni, 2005; Szebeni et al., 2011).

Previously it has been shown that C activation by liposomes can be caused by a negative charge on the phospholipid anchor molecule of PEG at the vesicle surface (Moghimi et al., 2006). In an attempt to avoid CARPA, alternative structures for PEG have been developed, like poly(amino acid)s, poly(glycerol) and poly(acrylamide), though these alternative surface structures...
appeared also to induce C activation (Knop et al., 2010; Romberg et al., 2007). To our knowledge, the only long-circulating liposomal formulation that has shown not to activate the C system is a very small-sized (<70 nm) DSPC:CHOL (2:1) liposomal formulation (Szebeni et al., 2002). However, a major disadvantage of these small liposomes is their low internal volume, resulting in a low encapsulation efficiency of water-soluble drugs (Knop et al., 2010).

In 1998, Bradley et al. reported that increasing both the PEG chain length and the molar concentration of PEG at the membrane effectively reduces C1q binding, and thereby reduces C activation via the classical pathway (Bradley et al., 1998). This to us was an indication that by changing the properties of the liposomal PEG-layer, the chance of C activation, and, hence, infusion reactions can be minimized while preserving the long circulating profile. The properties of the PEG layer can be altered by changing the PEG chain length, the anchor molecule for PEG in the liposomal bilayer and the grafting density of PEG. Since the severity of C activation is also known to be affected by liposomal size, lamellarity, charge, cholesterol content and the encapsulated drug (Bradley et al., 1998; Szebeni et al., 2012), we also investigated the influence of liposomal size, surface charge, and the presence or absence of encapsulated drug (prednisolone disodium phosphate (PLP)).

Several in vitro experiments were performed wherein we measured various C split products, each giving different information on the activation process. Thus, the SC5b-9 enzyme-linked immunosorbent assay (ELISA) assay measures the soluble, non-lytic form of the terminal C complex (TCC), is the end product of activation of the whole C cascade. It is generated by the assembly of C C5–C9 and subsequent binding to the regulatory S protein (Müller-Eberhard, 1984). The C system is activated via three different routes: the classical, lectin and alternative pathways. Activation of all separate pathways results in activation of the terminal pathway, resulting in formation of SC5b-9 (Kirschfink and Mollnes, 2003). Under normal conditions, activation of all three C pathways results in the formation of C3a from C3, which is also quantified by an ELISA. In the classical and alternative pathways, C3 activation results in the formation of C4d and Bb, respectively, which are quantified by a separate ELISA method, to allow quantitative assessment of the extent of activation of the alternative pathway by the tested formulations.

2. Materials and methods

2.1. Preparation of liposomes

The liposomes were prepared using a film extrusion method (Amselel et al., 1993). Briefly, dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol conjugate-2000 (DSPE-PEG2000) (both from Lipoid GmbH, Ludwigshaven, Germany), 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethylene glycol conjugate-550 (DSPE-PEG550, Avanti Polar Lipids Inc., Alabaster, AL, USA), 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE), cholesterol-polyethylene glycol conjugate-2000 (CHOL-PEG2000) (both from NOF, Grobbendonk, Belgium) and cholesterol (BUFA, Uitgeest, The Netherlands) were dispersed in ethanol in molar ratios as described in Table 1. A lipid film was created by rotary evaporation at 65 °C. The lipid film was hydrated with an aqueous solution containing prednisolone disodium phosphate (BUFA, Uitgeest, The Netherlands) at a concentration of 139 mg/mL, or phosphate buffered saline (PBS) (B. Braun, Melsungen, Germany) in case of empty liposomes. The resulting coarse dispersion was downsized by multiple extrusion steps through polycarbonate filter membranes with pore sizes of 50 or 100 nm to a final pore size as mentioned in Table 1. The size was confirmed by Dynamic Light Scattering (DLS). Subsequently, the unencapsulated prednisolone sodiumphosphate (PLP) was removed by dialysis against PBS using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kD, with repeated changing of the dialysis medium.

All compounds used were of pharmaceutical (Ph. Eur) or highly pure (≥99%) grade and were used without any further purification.

Since it is the lipid bilayer that causes the C activation, the concentration of bilayer components was kept constant in the tested formulations. Therefore, the lipid content of the liposomal solutions was determined by HPLC and the liposomal solutions were subsequently diluted with PBS to a final total lipid concentration of 55 mM, as these concentrations resulted in successful in vitro analysis previously (Romberg, 2007; Szebeni et al., 2002).

2.2. Characterization of liposomes

2.2.1. Measurement of size and size distribution

The size and size-distribution (polydispersity index, PDI) of liposomes were determined by dynamic light scattering (DLS) with a Malvern ALV CGS-3 system (Malvern instruments Ltd., Malvern, Worcestershire, United Kingdom) with a scattering angle of 90° at 25 °C. Samples were diluted approximately 150 times using phosphate buffered saline (PBS) (B. Braun, Melsungen, Germany) before measurement.

2.2.2. Drug and phospholipid analysis

Prednisolone phosphate concentrations were determined by high performance chromatography with UV detection (HPLC-UV) using an 1100 series HPLC system consisting of a binary pump, Model G1312A, an autosampler Model G1367A and a UV-detector Model G1314A (all from Agilent technologies, Amstelveen, The Netherlands). A Zorbax Eclipse-XDB-C8 analytical column (750 × 4.6 mm ID, particle size 5 µm, Agilent Technologies, Palo Alto, California, USA) preceded by a guard column (reversed phase 10 × 3 mm, Varian, Palo Alto, California, USA) were used. Absorbance was measured at 254 nm. Injection of 10 µL of sample was followed by a linear gradient of 5–90% acetonitrile (Bisolv B.V., Amsterdam, The Netherlands) with 10 mM ammonium formate (Fluka via Sigma–Aldrich, St. Louis, MO, USA). The pH was set at 3.6 using perchloric acid (Merck, Darmstadt, Germany). The flow rate was 1.0 mL/min. Chromatograms were processed using Chromeleon software ( Dionex Corporation, Sunnyvale, CA, USA).

To determine the amount of (un)encapsulated prednisolone disodium phosphate, an additional dialysis step was performed against PBS using Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Etten-Leur, The Netherlands) with a molecular weight cut-off of 10 kD. A 2 mL sample of the formulation solution was dialyzed against 600 mL of PBS for at least 8 h. Both the permeate and the retentate were analyzed on the above mentioned HPLC-UV system.

Lipid concentrations of the separate lipid components were determined by HPLC with evaporative light scattering detection (ELSD) using an 1100 series binary HPLC pump, Model G1312A (Agilent technologies, Amstelveen, The Netherlands), AS 3000 autosampler (Thermo Separation Products, Breda, The Netherlands) and an AltTech Varex MKIII Evaporative Light Scattering Detector (ELSD) (Grace (Alttech), Deerfield, IL, USA). An X-Bridge C18 analytical column (750 × 4.6 mm ID, particle size 2.5 µm, Waters corporation, Milford, MA, USA) was used. Injection of 30 µL of sample was followed by a linear gradient of 80–100% methanol (Bisolv B.V., Amsterdam, The Netherlands) with 1% triethylamine (Merck, Darmstadt, Germany). The flow rate was 0.4 mL/min. Chromatograms were processed using Chromeleon software.

Prior to HPLC analysis the samples were diluted to a concentration of approximately 1 μg/mL prednisolone phosphate or 3 mg/mL total lipid. Subsequently, an extraction using dichloromethane (Merck, Darmstadt, Germany), sterile water for injections (B. Braun) and methanol (Biosolve) was performed on the lipid-containing samples, to separate the prednisolone phosphate and the lipid compounds.

### 2.3. Measurement of surface charge (zeta-potential)

To determine the zeta-potential, the liposomal formulations were diluted 1:100 in 10 mM HEPES buffer. These diluted samples were measured by Laser Doppler Micro-Electrophoresis, using a Zetasizer Nano-Z (Malvern Instruments Ltd., Worcestershire, United Kingdom).

### 2.3. In vitro complement assays in human serum samples

#### 2.3.1. SC5b-9, ELISA

Whole blood samples of 10 healthy volunteers were collected in 10.0 mL BD Vacutainer® silicon coated glass serum tubes with no additives (BD, Franklin Lakes, NJ USA). Blood samples were allowed to clot at room temperature and subsequently centrifuged at 3000 rpm for 5 min to collect serum. Serum samples were aliquoted and stored at -20 °C. Frozen samples were rapidly thawed at 37 °C and kept on ice until use.

Complement activation was assessed by MicroVue SC5b-9 Plus ELISA kits (Quidel Co., San Diego, CA, USA). Serum from healthy volunteers was incubated with the diluted (55 mM total lipid) liposomal formulations (4:1) in duplicate for 30 min at 37 °C, as this concentration resulted in successful in vitro analysis previously (Romberg, 2007; Szebeni et al., 2002). After incubation the samples were diluted 20-fold in the “sample diluent” of the kit and 100 μl aliquots from this mixture were applied into the wells of the ELISA plate. The assays were performed according to the manual supplied with the kit. The absorbance was measured using a Wallac 1420 Victor 96-wellplate reader (Perkin–Elmer, Waltham, MA, USA) at 450 nm. SC5b-9 concentrations were calculated using a linear curve fit.

All formulations were tested in serum of 10 different individuals. In this article, the percentage of increase in complement activation marker formation compared to PBS (0% activation level) are used to quantify the activation of the complement system. Chan-an-Khan et al. previously showed high specificity and sensitivity of SC5b-9 as a biomarker of Doxil®-induced hypersensitivity reactions in cancer patients, but only when a more than 2-fold elevation of SC5b-9 levels compared to the baseline SC5b-9 level (0.25 ± 0.12 μg/mL (mean ± SD, n=50) (Buyon et al., 1992)) was seen. Based on sensitivity and specificity, these data suggest that only more than 2–4-fold (100–300%) increases of SC5b-9 levels can be taken as an elevated risk for the occurrence of hypersensitivity reactions, while a more than 4-fold (or 300%) increase is considered clinically relevant for the occurrence of hypersensitivity reactions (Chan-an-Khan et al., 2003). Therefore, we considered a 100–300% (2–4-fold) increase of the SC5b-9 concentration compared to the PBS baseline to be an increased risk on hypersensitivity reactions. In this line of thought, a more than 300% increase in SC5b-9 concentration is considered clinically relevant complement activation, with a high risk for hypersensitivity reactions in the patient. Below 100% and above 30% increase is considered to represent a mild activation of the complement system.

A schematic representation of the ELISA assay principle is given in Fig. 1.

#### 2.3.2. Other complement activation markers

C3a, C4d and Bb concentrations were determined by Microvue C3a Plus ELISA kits, Microvue C4d Plus ELISA kits and Microvue Bb Plus ELISA kits respectively (Quidel Co., San Diego, CA, USA). Serum samples of the 5 most sensitive individuals (as selected from the SC5b-9 concentrations formed) were incubated with the diluted liposomal formulations (4:1) for 30 min at 37 °C in duplicate. Incubated serum samples were diluted 5000-fold, 70-fold and 20-fold for the C3a, C4d and Bb analysis respectively, in
the supplied “sample diluents” of the kit and 100 µl aliquots from this mixture were applied into the wells of the ELISA plates. The assays were performed according to the kit manuals. The absorbance was measured using a Wallac 1420 Victor 96-wellplate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. C3a, C4d and Bb concentrations were calculated based on the calibration curves.

3. Results

3.1. Characteristics

The liposomal characteristics of the prepared liposomes are given in Table 2. HPLC analysis of the lipid components confirmed the molar lipid ratio of the liposomes as shown in Table 1. Increased PLP concentrations were found upon an increase of the liposomal size, which is a general observation with water-soluble drugs that are (passively) encapsulated in the aqueous interior of the liposome.

3.2. In vitro complement activation

First, the formation of the SC5b-9 complex was determined in sera of 10 separate individuals in duplicate. All liposomal formulations induced the formation of SC5b-9 to some extent in a number of sera. Most of these are considered to be mild biologically irrelevant activations, since the SC5b-9 concentrations have increased only 30–100% as compared to incubation with PBS, and are not considered statistically significant different (p > 0.05). However, the formulation in which PEG2000 was coupled to cholesterol instead of DSPE showed a significant (p < 0.05) 300–1600% increase in SC5b-9 concentration in sera of all tested individuals, indicating that this formulation induced relatively strong C activation (Fig. 2, formulation J). This was confirmed by C3a and Bb analysis, which showed increases of 1100% and 100%, respectively (Fig. 2, formulation J).

Formulations containing different amounts of DSPE-PEG were compared regarding their ability to activate the C system. As can be seen from Fig. 2, no significant changes in C activation were seen when the PEG density at the liposomal surface was reduced. With 2.5 mol% of PEG2000 at the liposomal surface (Fig. 2, formulation I) instead of 5 mol% (Fig. 2, formulation E), a comparable mean level of SC5b-9 formation, as well as a comparable number of sera showing (mild) increase of C3a formation were found. Nevertheless, serum of one individual showed >300% increase of C3a formation after incubation with the formulation with lowered surface concentration of PEG2000 (Fig. 2, formulation I), which might be suggestive of a higher risk of hypersensitivity reactions at lower grafting density of PEG, at least in some human sera.

It was also hypothesized that liposomes with a shorter PEG-chain could cause more activation of the C system. Therefore, formulations were prepared containing PEG550 instead of PEG2000. As can be seen from Fig. 2 (formulation E and G) reducing the PEG chain lengths to PEG550 did not result in a significant increase in the formation of SC5b-9 and C3a.

It was expected that an increase in liposomal size could increase C activation marker formation. However, the mean levels of both C3a and SC5b-9, as well as the number of sera of individuals showing (mild) activation, did not significantly change (Fig. 3). An approximately 300% increases of C3a formation as compared to PBS was seen in serum of the same individual for both the smallest (80 nm) and the largest size (120 nm) tested (Fig. 3). The same was found when comparing different sizes of PEG550 formulations (Formulation G and H, data not shown). Only the largest liposomal size tested (120 nm) showed a significantly increased activation of Bb (Fig. 3), indicating that size might be important for C activation by triggering the alternative pathway.

PEGylated liposomes containing PLP tend to show increased C activation as compared to their empty liposomal counterparts (Fig. 4). However, this increase of mean levels of C activation markers is only significant for PEG550 liposomes in the Bb assay. For two of the tested formulations, namely 100 nm PEG2000-liposomes with PLP and 100 nm PEG550-liposomes with PLP (formulations A and F respectively), serum of only one individual revealed a >300% increase of C3a levels versus PBS. Overall, these observations suggest a limited, pathway-specific impact of intraliposomal PLP on C activation only in some sensitive individuals’ sera.

![Fig. 2. Formation of complement activation markers after incubation of human serum with 100 nm empty liposomes with 5 mol% DSPE-PEG2000 (E), 5 mol% DSPE-PEG550 (G), 2.5 mol% DSPE-PEG2000 (I) and 5 mol% CHOL-PEG2000 (J).](http://dx.doi.org/10.1016/j.ejps.2013.03.007)
Considering the uniquely increased C activation by CHOL-PEG2000 liposomes (formulation J), we wished to explore the inter-experimental variation of increased C activation, as well as the role of classical pathway activation in it. To this goal, this formulation was re-tested in 5 reactive sera, measuring by ELISA the formation of SC5b-9, Bb, and C4d, taking the non-activating DPPC/C3 = significant complement activation marker formation as compared to the other formulations mentioned (p < 0.05).

**Fig. 3.** Formation of complement activation markers after incubation with liposomes increasing in size. Human serum was incubated with PLP-loaded liposomal formulations containing 5 mol% DSPE-PEG with sizes of approximately 80 nm (A), 100 nm (B), and 120 nm (C). Exact composition of the liposomes is given in Table 1. Formation of the different complement activation markers is given as % as compared to incubation with PBS (PBS = 0%). Each black dot represents the mean of a duplicate measurement of the complement activation marker in serum of 1 individual. Empty dots represent the mean of all sera, error bars represent the standard deviation. * = significant complement activation marker formation as compared to the equivalent empty formulation (p < 0.05).

**Fig. 4.** PEGylated liposomes with PLP tends to increase activation of the complement system compared their empty counterparts, as was shown for liposomes containing 5 mol% DSPE-PEG2000 of 80 nm (A) with encapsulated PLP and D without PLP) and 100 nm (B with encapsulated PLP and E without PLP) respectively, as well as for 100 nm liposomes containing 5 mol% DSPE-PEG550 (F with encapsulated PLP and G without PLP). Each black dot represents the mean of a duplicate measurement of the complement activation marker in 1 individual. Empty dots represent the mean of all individuals, error bars represent standard deviations. * = significant complement activation marker formation as compared to the equivalent empty formulation (p < 0.05).
PEG2000-DSPE liposomes (formulation B) and Zymosan as negative and positive controls. For these liposome preparations we also measured the zeta-potential, which resulted $-18.0 \pm 1.2$ and $-1.2 \pm 0.3$ mV (mean $\pm$ SD, $n = 4$) for formulations B and J, respectively.

As shown in Fig. 5, the major rise in the formation of SC5b-9 and Bb caused by CHOL-PEG2000 (J) liposomes vs. DSPE-PEG2000 (B) was reproduced, while C4d showed no difference among these liposome preparations. These findings confirm the major reduction of surface negativity by replacing DSPE with CHOL as PEG anchor, as well as the major increase of C activating power of these near-neutral CHOL-PEG2000 liposomes relative to the negatively charged, or otherwise near equivalent (DSPE-PEG2000) liposomes. Furthermore, these data provide strong evidence for that the excessive C activation of CHOL-PEG2000 liposomes proceeds mainly on the alternative pathway, without involving the classical pathway, at least to a major extent.

4. Discussion

The objective of this study was to investigate if changing the properties of the PEG layer on the liposomal surface reduces C activation that has been associated with the hypersensitivity reactions upon infusion of PEGylated liposomes (Chanan-Khan et al., 2003; Szebeni et al., 2002, 2012). Therefore, we measured the concentration of several C activation markers that were formed in vitro after incubation with liposomal formulations with different PEG layer properties.

Four C activation markers were selected that are known markers of C activation in different routes and stages. Specifically, C3a is a measure of C3 activation, Bb reflects activation of the alternative pathway, C4d the classical pathway and SC5b-9 provides a measure of the whole cascade until TCC. The sera of the individuals in this study were first screened for SC5b-9 formation since this factor is known to be a sensitive and consistent measure of C activation in human blood (Zsebeni, 2012; Szebeni et al., 2003).

Previous studies have shown that more than 2–4-fold (100–300%) increases of SC5b-9 levels in vitro vary assays may be considered as an elevated risk for the occurrence of HSRs in vivo, while a more than 4-fold (or 300%) increase is considered a clinically relevant risk for the occurrence of these reactions (Chanan-Khan et al., 2003). Since all formulations, except the CHOL-PEG2000 formulation showed only mild mean levels of C activation (less than 100% increased mean concentrations of SC5b-9 as compared to PBS), our data suggest that the PEGylated liposomal formulations studied here have low risk for causing HSRs in man. However, occasional reactions cannot be excluded, as shown by the higher values of C activation (exceeding 100% increase in SC5b-9 concentrations) in sera of specific individuals in our study.

Extensive research has been published concerning the occurrence of HSRs after administration of (PEGylated) liposomes. Various groups have shown that factors such as multilamellarity, a large size (>200 nm), a non-circular shape, a high cholesterol content (>71%) a high infusion rate (or bolus injection) all significantly increase the chance of C activation (Moghim et al., 2011; Szebeni et al., 2000). Previously it was thought that addition of PEG to the liposomal surface would reduce the interaction with plasma proteins and thus with C factors (as was shown in vitro for C1q) in the systemic circulation (Bradley et al., 1998; Szebeni et al., 2000). However, PEGylated liposomal products, like Doxil®, have been shown to induce C activation-related hypersensitivity reactions in patients (Chanan-Khan et al., 2003). Complement activation by liposomes in general has been related to the presence of an anionic phosphate moiety in the PEG-lipid conjugate, though the mechanism of the phenomenon remains elusive (Moghim et al., 2011). An alternative for PEG without this effect has not yet been found, and therefore PEG still remains the golden standard in the formulation of long circulating liposomes (Knop et al., 2010; Romberg, 2007; Romberg et al., 2007). In this study, it was found that a reduction of PEG chain length caused no significant changes in the activation of the C system in vitro, nor did a reduction of the PEG density at the surface, at least until 2.5 mol%.

Previously it has been shown for Doxil®, that the presence of doxorubicin influences the activation of the C system (Szebeni et al., 2002, 2012). It was hypothesized that the activation could be caused by a change in physicochemical properties of the liposomal surface due to the formation of elongated doxorubicin crystals in the liposomal aqueous core. In our study however, there also seems to be a tendency to an increased formation of C activation markers after loading the PEGylated liposomes with PLP, which is encapsulated as a solution in the liposomal aqueous interior. We cannot exclude that PLP and other drugs that are encapsulated in dissolved form rather than as crystals, can also cause slight changes to the liposomal formulation that result in an increased chance of C activation. This warrants a more in depth investigation of the physicochemical characteristics of PLP-PEGylated liposomal formulations.

The formulation in which cholesterol was used as the anchor molecule for PEG, instead of normally used DSPE, showed unexpectedly strong C activation. Although unsuitable as a potential new drug delivery vehicle in view of the activation results, this formulation might provide a new tool for better understanding the mechanism of C activation by PEGylated liposomes. CHOL-PEG2000 differs from DSPE-PEG2000 in that the former has no negatively charged phosphate group in its lipophilic anchor that stabilizes the position of the PEG-lipid-conjugate relative to the membrane surface. Because this charged phosphate group appeared to be the main cause of C activation by PEG-DSPE (Moghim et al., 2006), it was assumed that replacing it with another, not charged, or less charged PEG carrier might reduce the risk of C activation. However, the opposite was found as CHOL-PEG2000 liposomes show reactivity for CHOL-PEG2000 (J) liposomes vs. DSPE-PEG2000 (B) and J, respectively. Error bars represent standard deviations ($p < 0.05$).

Fig. 5. Complement activation by CHOL-PEG2000 (J) and DSPE-PEG2000 (B) liposomes in normal human sera that showed reactivity for CHOL-PEG2000 liposomes (see Fig. 2, formulation J) relative to PBS (PBS = 100%). Experimental procedures were the same as in Figs. 2–4, except that zymosan (5 mg/mL) was also used as positive control. Error bars represent standard deviations ($n = 5$). * = significant rise of activation marker (SC5b-9, Bb and C4d) compared to the non-activating formulation B ($p < 0.05$).
In summary, we found that PLP containing PEGylated liposomes are weak C activators in some, but not all human sera. This suggests a relatively small, but not negligible risk for infusion reactions mediated by autoantibodies to cholesterol in miniature pigs. J. Immunol. 118, 342–347.


