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Liposome-induced activation of the classical complement pathway does not require immunoglobulin

Jihan Marjan, Zongcen Xie, Dana V. Devine *

Department of Pathology, University of British Columbia, 2211 Wesbrook Mall, Vancouver, B.C. V6T 2B5, Canada and Canadian Red Cross Blood Transfusion Service, Vancouver, B.C., Canada

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Abstract

We have investigated the contribution of immunoglobulin to the liposome-induced activation of complement in human serum. Liposomes containing the negatively charged phospholipids cardiolipin, phosphatidylglycerol or phosphatidylinositol, in addition to phosphatidylcholine and cholesterol, were used to activate complement in a whole serum system. The contribution of immunoglobulin was studied by comparing normal human serum (NHS) to serum depleted of IgG and IgM (DDS). Using hemolytic assays of complement function, greater concentrations of phospholipids were required to activate complement in the absence of immunoglobulins. Activation of the classical pathway was confirmed using a C1q ELISA which showed that activation was dependent on the presence of C1q and confirmed that greater concentrations of phospholipids were required to activate complement in the absence of immunoglobulins. Complement activation was also assessed using crossed immunoelectrophoresis of C3 activation fragments. Using immunoblot analysis, iC3b was detected on the surface of liposomes exposed to NHS or DDS. These studies demonstrate that when liposomes, containing anionic phospholipids at an equivalent charge to cardiolipin 20 mol%, are exposed to immunoglobulin depleted serum they become opsonized by complement proteins.

Key words: Liposome; Complement activation

1. Introduction

Phospholipid vesicles known as liposomes have potential promise as packaging systems for drug delivery [1–5]. In this setting, they interact with plasma proteins including those of the complement system. In vitro studies [6–9] have shown that liposomes interact with blood proteins, some of which could contribute to their clearance by phagocytic cells in vivo. Thus, the activation of complement by liposomes can result in the opsonization of the liposomes for phagocytic clearance by cells of the reticuloendothelial system. Liposomes may activate the classical or the alternative pathway depending on the physicochemical properties and type of lipid used [10–12]. The activation of the complement system by liposomes has been described for only a few specific lipid compositions. These compositions include lipids conjugated to haptens [13], phosphatidylserine with phosphatidylethanolamine [14], cardiolipin (CL) [15], sterylamine in the presence of galactosylceramide [16], cerebrosides in dimyristoyl-phosphatidylethanolamine [17] or saturated phosphatidylethanolamine in saturated phosphatidylcholine (PC)-containing cholesterol [18]. These reports do not define any fundamental property of complement-activating liposomes. We have previously demonstrated that negatively charged liposomes activate the classical pathway in normal human serum while those that are positively charged activate the alternative pathway [19]; therefore, charge-bearing phospholipids fall into two distinct groups with respect to complement activation.

In most circumstances, activation of the classical pathway occurs following the binding of an antibody to

* Corresponding author. Fax: +1 (604) 8227575.

Abbreviations: PI, phosphatidylinositol derived from plant; PC, phosphatidylcholine derived from egg; PG, phosphatidylglycerol derived from egg; CL, cardiolipin; CH, cholesterol; CH*, cholesterol [^{3}H] hexadecyl ether; VBS, veronal buffered saline; EDTA, ethylenediamine-tetraacetic acid, disodium salt; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DDS, IgG- and IgM-depleted serum.

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its antigen. Specifically, the immunoglobulins IgG and/or IgM bind to the antigenic surface followed by the binding of the first component of the complement pathway, C1q, to the Fc portion of the antibody. Naturally occurring antiphospholipid antibodies that activate the classical complement pathway have been reported in humans and animals [20–23].

Activation of the classical pathway can also occur in the absence of specific antibody and has been well-documented for a number of charged particles including urate crystal [24], DNA and RNA [25], Gram-negative bacteria [26], retroviruses [27] and heparin protamine [28]. In addition, using a purified protein system, Kovacsovics et al. [15] demonstrated direct binding of C1q subunits with subsequent activation of complement component C1 by cardiolipin-containing liposomes, a finding which was reported to be unique to cardiolipin. Two factors have been identified to be important in the antibody-independent binding of C1q, namely the presence of multiple binding sites in order to bind the multiple heads of the C1q molecule [29] and the presence of a negative surface charge [15,30].

In this study, we have addressed the role of immunoglobulin in the activation of complement by negatively-charged liposomes in the presence of all serum proteins with the exception of complement-activating immunoglobulins. Using functional assays and activation fragment analysis, we demonstrate that C1q binds to all negatively-charged lipids tested and that the binding induces further activation of the complement pathway through an antibody-independent mechanism which results in opsonization of the liposomes.

2. Materials and methods

Preparation of liposomes. Multilamellar vesicles were prepared according to established methods [31]. Large unilamellar vesicles were prepared by extrusion of multilamellar vesicles through 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver, Canada) [32,33]. The lipids used in this study were purchased from either Avanti Polar lipids (phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylinositol (PI)) or from Sigma (cholesterol (CH)). These lipids were used without further purification. The liposomes were resuspended in isotonic VBS buffer (10 mM sodium barbital, 145 mM NaCl (pH 7.4)). The average diameter of the liposomes was 105 ± 19 nm ($n = 6$) for PC:CH:CL (35:45:20 mol%), 106 ± 16 nm ($n = 6$) for PC:CH:PG (15:45:40 mol%), 98 ± 7 nm ($n = 6$) for PC:CH (55:45 mol%), 101 ± 10 nm ($n = 6$) for PC:CH:PI (15:45:40 mol%) and 110 ± 10 nm ($n = 6$) for CH:PG (45:55 mol%) as determined by quasielastic light scattering and NICOMP (model 270) analysis using a vesicle Gaussian unimodal distribution mode. The addition of anionic phospholipid at the concentrations indicated did not affect the liposome size. The phospholipid content was measured using a colorimetric assay [34].

Preparation of normal human serum and immunoglobulin-depleted serum. The normal human serum pool (NHS) prepared from venous blood from 27 healthy individuals (15 female and 12 male) was allowed to clot at room temperature for 30 min and centrifuged at $275 \times g$ at 4°C for 10 min to remove red and white cell contaminants prior to storage at −70°C. To prepare serum depleted of immunoglobulin classes IgG and IgM (double depleted serum; DDS), serum was passed through an anti-IgM-Sepharose affinity column; the anti-IgM affinity column was prepared by coupling anti-human IgM antibody (Zymed Labs, San Francisco, CA) to CNBr-Sepharose 4B according to established methods [34]. The eluate obtained was subsequently passed though a Protein G-Sepharose column to remove all subclasses of IgG. The levels of IgG and IgM were quantitated using a QM 300 Nephlometer (Sanofi Diagnostics, Chaska, MN). In NHS at a protein concentration of 12 mg/ml, the level of IgG was 3.3 g/l and that of IgM was 0.323 g/l; undiluted NHS contained 11.3 g/l and 1.15 g/l of IgG and IgM, respectively and was within normal acceptable range. In DDS at a protein concentration of 12 mg/ml the levels of IgG and IgM were below the lower limit of detection of the assays and were therefore reported as < 0.069 g/l and < 0.082 g/l, respectively. Reducing and non-reducing SDS-PAGE of NHS and DDS (both at a final protein concentration in reaction mixture of 12 mg/ml) revealed that immunoglobulins were the only bands missing from protein profiles (data not shown). Specific depletion of IgG was confirmed, as all bands detected in NHS were also detected in DDS with the exception of those bands corresponding to IgG. A sample of pure IgG was run on the same gel to confirm that the molecular weight of the depleted protein corresponded to that of IgG. The depletion of IgG was also confirmed by Western blot analysis under non-reducing conditions; a 150 kDa band reactive with anti-IgG in NHS was no longer present in DDS. Similarly, the depletion of IgM was confirmed by Western blot; the 970 kDa band reactive with peroxidase-conjugated anti-IgM in NHS was no longer present in DDS. In all experiments described below, DDS was concentrated in 10 kDa cutoff microconcentrators (Filtron, Northborough, MA) to 12 mg/ml for all assays except the C1q ELISA which was used at 6 mg/ml. NHS was used at equivalent protein concentrations as determined by BioRad Protein Assay (BioRad, Richmond, CA).

Using the C1q ELISA described below, we found that the C1q level was markedly reduced in DDS compared to NHS ($A_{620\,nm} = 0.02$ and 0.27, respectively). Immunoblot analysis of C1q content in NHS and DDS indicated near complete depletion of C1q from DDS during the removal of immunoglobulins from NHS (data not shown). Therefore, DDS was repleted with C1q (Quidel, San Diego, CA) to normal serum concentrations of 0.063 mg/ml. Manufacturer's analysis of C1q indicates that the purified material is greater than 95% pure and retained full function. The final pool of highly purified C1q was tested for the presence of contaminating immunoglobulin proteins by double immunodiffusion, immunoelectrophoresis and ELISA procedures and was found to be free of immunoglobulins.

Along with C1q, $CaCl_2$ and $MgCl_2$ were added to DDS after all manipulations of the serum were completed, to a final concentration of 0.15 mM and 1 mM, respectively. The manipulations to convert NHS to DDS and the additions of the cations to DDS did not alter its hemolytic activity in either the alternative (APH50) or the classical complement pathway (CH50) assays. Standard CH50 and APH50 analyses were performed by the University Hospital Department of Laboratory Medicine, Vancouver, B.C. The reported value for NHS was 150 U/ml in the CH50 and 162 U/ml in the APH50 while DDS contained 200 and 125 U/ml, respectively. The normal range for the CH50 is 114–202 U/ml and 99–173 U/ml for the APH50; therefore, the normal and immunoglobulin-free sera used in these experiments fell within the clinical normal range for complement function. Thus, analysis of the reagents used in the studies described below confirms the absence of immunoglobulin IgG and IgM from DDS as well as the adequate replacement of C1q and required divalent cations.

Functional complement assay (CH50). The functional complement assay (CH50) was slightly modified to measure residual complement activity in serum that had been exposed to liposomes. Liposomes were titrated serially in VBS containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, 5% D-glucose, 0.1% gelatin ($DGVB^{2+}$) and 100 μl aliquots of NHS or DDS at 12 mg/ml was added to each dilution. The liposome-lysis control (color blank) consisted of liposomes incubated in the absence of serum and was analyzed in parallel with the test samples at an equivalent liposome dilution; the absorbance of these samples at 414 nm was always < 0.004. The amount of complement activity remaining in each tube was compared with that of serum incubated in the absence of liposomes (100%). The contribution of serum alone to the absorbance values of the test samples was $< 2\%$ of the maximum lysis. 200 μl of each sample were then incubated for 30 min at 37°C, then diluted up to 400 μl using ice-cold $DGVB^{2+}$ and kept on ice. The total residual complement content of liposome-treated serum was measured by hemolytic assay (CH50) using established methods as described by Whaley [35]. Briefly, sheep erythrocytes were sensitized with rabbit IgM anti-sheep RBC antibody (EA) and suspended at a concentration of 10^9 cells/ml in $DGVB^{2+}$. 50 μl of EA were incubated with 50 μl of liposome-treated serum for 30 min at 37°C. Samples were then diluted by adding 1 ml VBS containing 0.1% gelatin and 20 mM EDTA (GVB-EDTA). Unlysed EA cells were pelleted by centrifugation and the amount of hemoglobin released into the supernatent was quantitated spectrophometrically at 414 nm. The percent lysis of EA targets was calculated for each liposome dilution as follows:

$$(\text{mean test} - \text{color blank at } A_{414\,nm} / 100\% - \text{color blank at } A_{414\,nm}) \times 100\%$$

The percent complement consumption by the liposomes, that is, the reduction in starting complement activity, was then calculated as 100 − % EA lysis.

Analysis of C1q binding to liposomes. This assay was based on the affinity of C1q for aggregated IgG. Whole human IgG (Accurate Chemical and Scientific, Wesbury, NY) was aggregated by heating at 62°C for 30 min. 130 μl of aggregated human IgG at 20 μg/ml was then adsorbed to a 96-well Immunolon II microtitre plate (Dynatech Labs, Chantilly, VA) at 4°C overnight. Unreacted protein binding sites were blocked with PBS containing 3% BSA. Liposomes were titrated serially in VBS and reacted with an equal volume of NHS or DDS at a protein concentration of 6 mg/ml at 37°C for 30 min. After this period the microtitre plate was washed three times with PBS containing 0.5% BSA and 0.5% Tween-20. The liposome-treated serum mixtures (130 μl) were then added to the microtitre plate wells and incubated at 37°C for 1 h. Maximum levels of C1q were detected in wells containing either NHS or DDS but no liposomes (100%): the negative controls consisted of liposomes incubated in the absence of serum for each dilution used. The residual binding of C1q to aggregated IgG was proportional to the level of free C1q in the serum. C1q binding was detected by incubating with a primary goat anti-human C1q antibody (Quidel, San Diego, CA) and subsequently with peroxidase-labelled rabbit anti-goat IgG. Both antibodies were diluted to the appropriate titre in PBS containing 3% (w/v) powdered skim milk. Bound peroxidase-conjugated antibody was detected using 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO) and the color measured at 620 nm using a spectrophotometer. The percent C1q binding to IgG after incubation with

liposomes was calculated for each liposome dilution as follows:

$$(\text{mean test} - \text{color blank at } A_{620\,nm} / 100\% - \text{color blank at } A_{620\,nm}) \times 100\%$$

The percent C1q consumption, that is, the amount bound to the liposomes, was then calculated as 100 – % C1q bound to IgG-coated plates.

C3 crossed immunoelectrophoresis. In this assay, 1% agarose in VBS was poured onto 5 × 5 cm glass plates. Undiluted samples (10 μl) were placed into sample wells cut 2 cm from the bottom of the glass plate. The plates were electrophoresed at 35 mA for 9 h. The gel was cut 0.25 cm above the sample well and an agarose layer containing polyclonal goat anti-human C3 antibody (Quidel) was added at 1:200 in VBS. Electrophoresis was then carried out at right angles to the original electrophoresis at 30 mA for 6–7 h. Afterwards, the plate was dried using absorbent paper and the C3-anti-C3 precipitate was visualized using Coomassie blue R-250 and then destained using 30% methanol solution. Negative controls consisted of NHS or DDS incubated with non-activating liposomes (PC:CH at 45:55 mol%); all liposome compositions were used at a final concentration of 25 mM. The positive control, in which maximum conversion of C3 is achieved, consisted of serum incubated with inulin, a potent activator of complement. For each of the liposome preparations tested, immunoprecipitation patterns were compared with that of the negative control. The treatment of serum with inulin results in the cleavage of C3 to C3b and iC3b. These smaller fragments migrate further from the origin than native C3 in the first dimension of electrophoresis and subsequently appear as a distinct peak separated from native C3 in the precipitin arc. The areas under the shifted curve were calculated using the Bioquant program (R and M Biometrics, Nashville, TN) from eight successive readings.

Immunoblot analysis of proteins associated with liposomes after serum incubations. Liposomes were trace labeled with cholesterol [^{3}H]hexadecylether (CH*), and incubated with undiluted NHS or DDS (1:4 v/v). Incubations proceeded at 37°C for 30 min after which the mixtures were passed through spin columns to separate liposomes from free serum components as described by Chonn et al. [33]. The radioactivity associated with each liposome composition was counted in a β scintillation counter and standardized before running on a gel. Protein separation was performed by SDS-PAGE under reducing conditions on precast 4–15% gradient resolving Phast gels (Pharmacia) using an automated electrophoresis apparatus, the Phast System (Pharmacia). Prestained SDS-PAGE standards (Diversified Biotech, Newton, MA) were used to estimate the molecular weight of the proteins separated. Proteins were transferred onto a nitrocellulose membrane using a mini trans blot electrophoretic transfer cell (BioRad). Unreacted protein binding sites were blocked overnight at room temperature using 5% (w/v) powdered skim milk in PBS-Tween (0.13 M NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 3.7 mM Na_2HPO_4 and 1% Tween-20). The nitrocellulose paper was then incubated with goat anti-human C3, anti-human factor B, anti-human C7 or anti-human C9 (Quidel) or non-immune goat serum diluted to 1:500 in the same buffer for 1 h at room temperature. After washing three times for 5 min in PBS containing 0.5% Tween-20, blots were then incubated with a peroxidase-conjugated rabbit anti-goat antibody (Jackson Immunoresearch, Avondale, PA) diluted to 1:5000 in PBS containing 5% (w/v) powdered skim milk and 1% Tween-20. Bound antibody was visualized using the Amersham ECL Western blot detection system (Amersham, UK).

Measurement of alternative pathway activation. To assess activation of the alternative pathway, two different methods were employed. First, the alternative pathway hemolytic activity was assessed using an alternative pathway CH50 assay (APH50). This assay is described in detail in [19] and is based on the same principle as the classical pathway functional assay described above. Briefly, serum exposed to liposomes (PC:CH:CL (35:45:20 mol%) at 12.5 mM final concentration, PC:CH:PG (15:45:40 mol%) and PC:CH:PI (15:45:40 mol%) both at 25 mM) was incubated with rabbit erythrocytes suspended in VBS containing 3.75% D-glucose, 0.03% gelatin, 7 mM $MgCl_2$ and 20 mM EGTA. In the presence of EGTA, rabbit erythrocytes activate the alternative pathway, but not the classical pathway; the degree of hemolysis is proportional to the functional alternative pathway activity in the sample. Alternative pathway activation was also measured using an ELISA for the activation fragment Bb (Quidel). This fragment is generated by the cleavage of factor B during alternative pathway activation. Microtitre plates coated with monoclonal antibody specific for the Bb fragment capture Bb from the serum sample. After washing, the bound Bb is detected using a peroxidase-conjugated goat-anti-Bb antiserum. The amount of colored reaction product was compared to that generated by standards of purified Bb including low (0 mg/l), medium (0.103 mg/l) and high (0.267 mg/l) standards. Several normal serum pools were used to establish a normal range; the Bb values for undiluted serum from healthy individuals were between 0–9 mg/l. Samples consisting of PC:CH:CL (35:45:20 mol%), PC:CH:PG (15:45:40 mol%) and PC:CH:PI (15:45:40 mol%) were exposed to NHS and DDS at a final concentration of 50 mM lipid and run in duplicate. A sample containing NHS was exposed to inulin in the presence

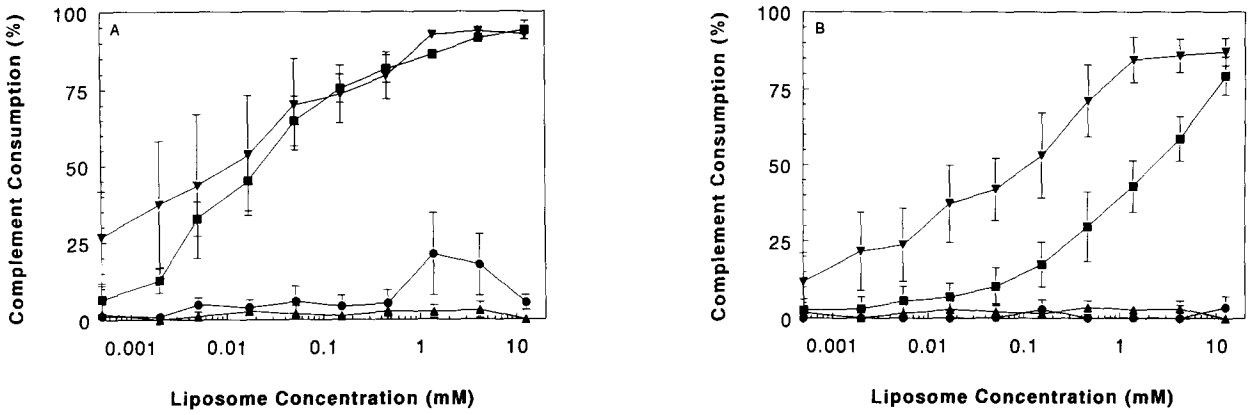


Fig. 1. Complement consumption in human serum by CL-containing liposomes. Large unilamellar vesicles containing varying amounts of CL were incubated with NHS (panel A) or DDS (panel B). Their effect on complement activity compared to the absence of liposomes was determined by complement hemolytic assays (see Section 2). The phospholipid vesicles were composed of PC:CH (55:45 mol%) (▲), PC:CH:CL (50:45:5 mol%) (●), PC:CH:CL (42.5:45:12.5 mol%) (■), or PC:CH:CL (35:45:20 mol%) (▼). Each point represents the mean of 4 experiments each done in duplicate; bars represent 1 S.E.

of 1 mM magnesium chloride; similar samples with DDS were prepared. The samples were incubated at 37°C for 1 h. Positive controls were prepared by incubating NHS or DDS with inulin to activate the alternative pathway and generate Bb fragments.

3. Results

To investigate the role of complement-activating immunoglobulin isotypes in liposome-induced complement activation, NHS was depleted of IgG and IgM by passing through an anti-IgM-Sepharose affinity column and subsequently through a Protein G-Sepharose column. No IgG or IgM was detected in DDS by nephelometry or immunoblot and the remaining protein components remained unchanged as determined by silver-stained SDS-PAGE (data not shown).

Fig. 1 demonstrates the effect of concentration of CL in the liposome composition on the activation of complement in NHS and DDS, respectively. Complement consumption in NHS was observed at 5 mol% CL and greater (Fig. 1A). In DDS, complement consumption was only observed when CL was present in at least 12.5 mol% (Fig. 1B). Fig. 2 demonstrates the effect of concentration of PG in the liposome on the activation

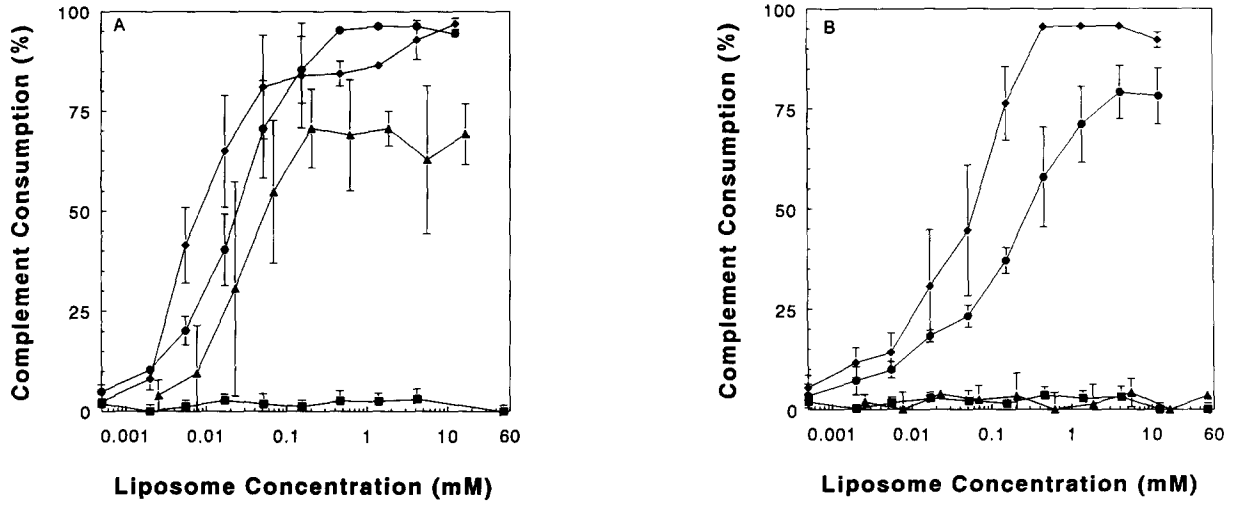


Fig. 2. Complement consumption in human serum by PG-containing liposomes. Large unilamellar vesicles containing varying amounts of PG were incubated with NHS (panel A) or DDS (panel B). Their effect on complement activity compared to the absence of liposomes was determined by complement hemolytic assays (see Section 2). The phospholipid vesicles were composed of PC:CH (55:45 mol%) (■), PC:CH:PG (35:45:20 mol%) (▲), PC:CH:PG (15:45:40 mol%) (●), or CH:PG (45:55 mol%) (♦). Each point represents the mean of 3 experiments each done in duplicate; bars represent 1 S.E.

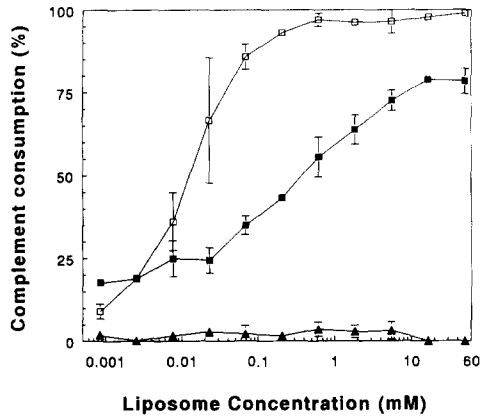


Fig. 3. Complement consumption in human serum by PI-containing liposomes. Large unilamellar vesicles containing various amounts of PI were incubated with NHS (open symbols) or DDS (closed symbols). Their effect on complement activity compared to the absence of liposomes was determined by complement hemolytic assays (see Section 2). The phospholipid vesicles were composed of PC:CH:PI (15:45:40) (squares) or PC:CH (55:45 mol%) (triangles). Complement consumption by PC:CH vesicles in DDS was indistinguishable from NHS. Each point represents the mean of 3 experiments each done in duplicate; bars represent 1 S.E.

of complement. Complement consumption was evident in PG 20 mol% and higher (Fig. 2A); however, complement consumption in DDS was not evident until PG was present at 40 mol% (Fig. 2B). The experiment was performed with a third anionic phospholipid, PI, and again complement consumption was evident in DDS when the anionic phospholipid was present at 40 mol% (Fig. 3). With all three anionic phospholipids, more lipid was required in DDS to observe the same level of complement consumption seen in NHS. The neutral-charged composition PC:CH (55:45 mol%) did not consume complement and was used as a negative control. Using a 3-way ANOVA comparing anionic phospholipid mol%, lipid concentration and immunoglobulin presence or absence (SYSTAT, Evanston, IL), each of the liposome compositions PC:CH:CL (35:45:20 mol%), PC:CH:PG (15:45:40 mol%) and PC:CH:PI (15:45:40 mol%) showed a statistically significant lipid concentration effect ($P < 0.01$), a significant increase in complement activation compared to net neutral compositions ($P < 0.001$) and a significant difference in the degree of activation when exposed to NHS versus DDS ($P < 0.01$).

In order to verify that the CH50 assay was measuring complement activation-induced loss of activity rather than passive adsorption of complement proteins onto the liposomes, complement activation was also monitored using crossed immunoelectrophoresis to measure C3 degradation products. Although the studies described below indicate that liposomes activate complement through antibody-independent classical pathway activation, inulin was used to generate the maximum conversion of C3 to degradation products C3b and iC3b as a positive control for the crossed immunoelectrophoresis. The cleavage products of C3 are identical irrespective of the pathway used to generate them. Results showed that liposomes exposed to immunoglobulin-depleted serum produced C3 degradation fragments, indicating that anionic phospholipids are able to activate complement in the absence of immunoglobulin (Table 1). Confirming the results of CH50 analyses, less degradation was measured in DDS than in NHS for a given amount of lipid. Crossed immunoelectrophoresis does not distinguish between C3b and iC3b; therefore, the relative proportions of C3 degradation products cannot be assessed by this method.

Table 1
Activation of complement detected by curve shift in crossed immunoelectrophoresis; area under the curve (mm^2) ± 1 S.D. of 8 values

Treatment	NHS	DDS
Inulin	3.075 ± 0.0767	3.279 ± 0.0864
EPG 55%	3.052 ± 0.137	1.280 ± 0.091
CL 20%	2.317 ± 0.0864	0.651 ± 0.124
Untreated	0	0

When alternative pathway activation by liposomes was assessed, no significant reduction in complement activity in the APH50 assay was seen with any of the liposome compositions tested (data not shown). To distinguish activation of the alternative pathway from passive adsorption of complement proteins required in the APH50 assay, factor Bb fragment levels were determined; Bb is an activation peptide generated during alternative pathway activation. There was no significant increase of Bb in any of the liposome compositions when exposed either to NHS or DDS at a final lipid concentration of 50 mM (Table 2). These data are also supported by the absence of native factor B or factor Bb fragments on the surface of these liposomes using Western blotting techniques (data not shown).

To further confirm that the activation in NHS and DDS proceeded via the classical pathway, the C1q ELISA was used to monitor the consumption of C1q

Table 2
Levels of the alternative pathway activation fragment Bb in NHS or DDS exposed to liposomes

	Bb level (mg/l) (Mean of 4 values ± 1 S.D.)	
	NHS	DDS
High serum control	13.8 ± 6.86	
Low serum control	0	
PC:CH:CL (35:45:20 mol%)	2.475 ± 2.475	5.2 ± 2.54
PC:CH:PG (15:45:40 mol%)	5.75 ± 1.25	4.35 ± 2.78
PC:CH:PI (15:45:40 mol%)	5.81 ± 0.89	6 ± 0.5
Inulin	> 13.8 [a]	> 13.8 [a]

[a] Highest level accurately read from standard curve.

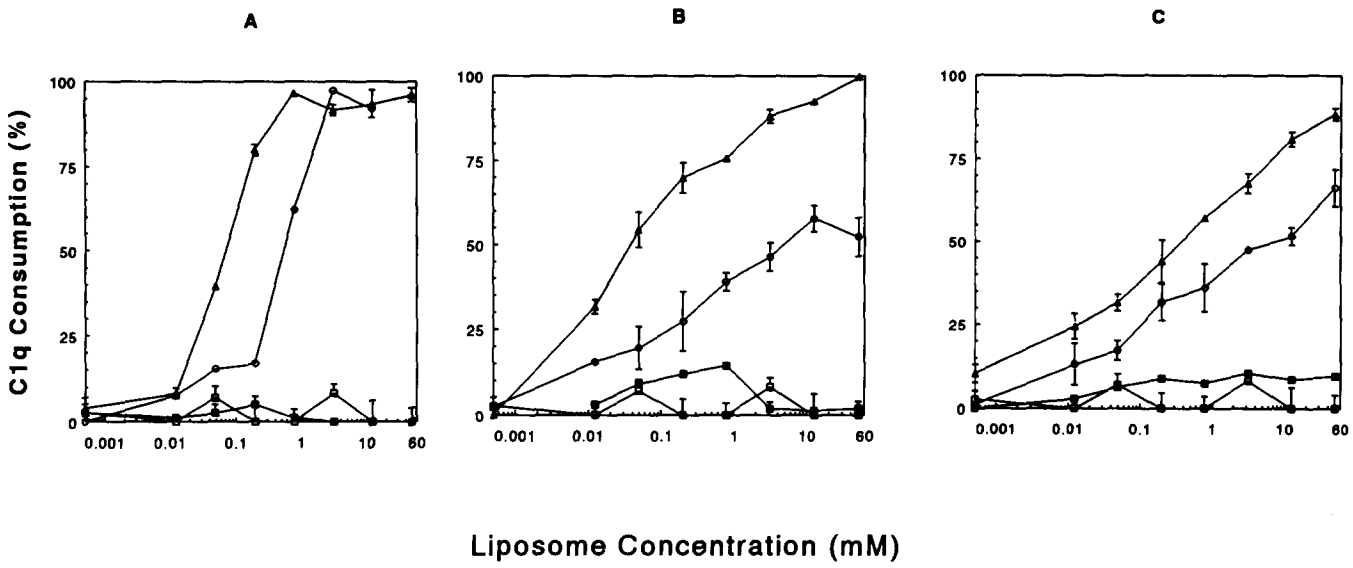
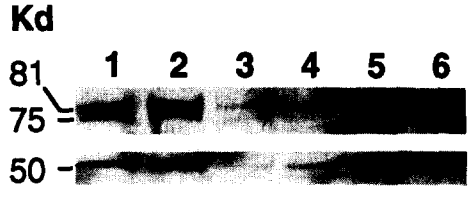


Fig. 4. Effect of immunoglobulin on C1q binding to liposomes containing anionic phospholipids. Liposomes composed of PC:CH:CL (35:45:20 mol%) (panel A), CH:PG (45:55 mol%) (panel B) or PC:CH:PI (15:45:40 mol%) (panel C), were incubated with NHS (▲), with DDS (○) or with DDS lacking C1q (■). Binding of C1q to PC:CH (55:45 mol%) liposomes incubated with NHS is shown in each graph (□). An ELISA was used to measure the residual C1q binding to aggregated IgG (see Section 2). Each point represents the mean of 4 experiments each done in triplicate; bars represent 1 S.E.

from NHS or DDS exposed to liposomes. C1q was consumed in a concentration-dependent manner following the incubation of liposomes containing CL at 20 mol%, PG at 55 mol% and PI at 40 mol% (Fig. 4) with NHS or DDS. Consumption did not occur in DDS that had not been repleted with C1q, confirming the removal of C1q during immunoaffinity chromatography, nor did it occur with the neutral composition PC:CH (55:45 mol%). In order to control for liposome adherence to the microtitre wells, liposomes containing PI at 40 mol% were trace-labeled with cholesterol [^{3}H]hexadecylether prior to incubation with serum in the plate as described in Section 2. Although C1q consumption could be readily measured by the ELISA, none of the wells exceeded background counts of 38 cpm ± 2%, indicating no adsorption of liposomes to the microtitre plates. Negative controls which included plates coated with BSA alone showed no C1q consumption (data not shown).

Fig. 5 shows the detection of iC3b on the surface of negatively-charged liposomes exposed to NHS or DDS. Exposure of liposomes composed of PC:CH:CL at 35:45:20 mol%, PC:CH:PI at 15:45:40 mol% and PC:CH:PG at 15:45:40 mol% to DDS followed by Western blot analysis resulted in the detection by anti-C3 of three bands at 81 kDa, 75 kDa and 50 kDa. These molecular masses correspond to the β chain (75 kDa) and the two fragments of the α chain of iC3b (50 and 81 kDa). Liposomes of the same compositions exposed to NHS also had iC3b on their surface. Exposure of liposomes composed of PC:CH (55:45 mol%) to NHS or DDS showed no bands at 50, 75 or 81 kDa (data not shown). NHS exposed to inulin to achieve maximum conversion for C3 to iC3b produced bands at 50, 75 and 81 kDa (data not shown). Blots probed with normal goat serum as the primary antibody failed to



Lane	1	2	3	4	5	6
50Kd	0.59	0.7	0.47	0.52	0.79	0.52
75Kd	0.6	0.6	0.46	0.51	0.78	0.55
81Kd	0.69	0.71	0.56	0.58	0.77	0.62

Fig. 5. Immunoblot analysis of iC3b bound to negatively charged liposomes exposed to NHS or DDS. Liposomes at 100 mM were exposed to serum and separated from free serum components by chromatography. Liposome samples were made equivalent for lipid content at 3.6 mM and were then run on a 4–20% gradient SDS-PAGE gel, transferred onto nitrocellulose paper and probed with goat anti-human C3 antibodies. Lanes 1, 3, and 5 contain liposomes exposed to NHS while lanes 2, 4, and 6 contain liposomes exposed to DDS. Liposomes were composed of PC:CH:CL (35:45:20 mol%) (lanes 1 and 2), PC:CH:PI (15:45:40 mol%) (lanes 3 and 4), or PC:CH:PG (15:45:40 mol%) (lanes 5 and 6). Bands at 50, 75 and 81 kDa corresponding to the β-chain and the two halves of the α chain of iC3b were detected. Densitometer readings of the Western blot are given in the table as absorbance units. No bands were detected when the blot was incubated with normal goat serum.

yield any bands. Densitometric analysis of the Western blots suggests that C3b deposition occurs to a greater degree with compositions containing PG or CL than in those containing PI as the source of anionic phospholipid (Fig. 5). Thus, complement was activated at least to the C3 step by the liposomes and the opsonizing complement component iC3b was deposited on the liposome surface. Western blots of liposomes probed with polyclonal antibodies to C7 failed to detect this complement protein on the surface of the liposomes (data not shown). This is likely due to the limit of detection of these assays, as we could demonstrate C9 associated with CL- or PG-containing liposomes both in these studies and in previous work [19].

4. Discussion

The data reported herein demonstrate that unilamellar phospholipid vesicles bearing a net negative charge activate the classical pathway in an antibody-independent manner. The absence of complement-activating immunoglobulins from an otherwise intact serum system did not block the ability of negatively charged liposomes to activate complement. We have previously demonstrated [19] that liposomes with exposed surfaces bearing negative charges activate the classical pathway of complement. In the studies reported herein, we provide evidence that classical pathway activation is independent of the presence of antiphospholipid antibodies or other complement-activating immunoglobulins, and that negatively charged liposomes can deplete C1q from serum while liposomes lacking a net surface charge do not.

Our observation of C1q-mediated activation of complement by cardiolipin-containing liposomes in the absence of antibody is supported by studies of Kovacsovics [15] which reported activation of purified ^{125}I-C1 by liposomes of similar composition. However, in those studies, cardiolipin was the only phospholipid which bound C1q; other negatively charged phospholipids such as PI showed no activation. In our experimental system, all negatively charged phospholipids tested induced complement activation by liposomes. The inconsistency between these two studies may be due to methodological differences. In the Kovacsovics study, phospholipids were used at 30–70 μM, depending on the composition. Thus, both the relative concentration and the theoretical net surface charge differed for liposomes containing PC, PE, or PI. Our data indicate that when the overall charge is the same, negatively charged phospholipids are similar in their C1q binding ability. This finding suggests that charge influences classical pathway activation by liposomes; however, the precise mechanism by which surface charge facilitates the activation of complement by liposomes remains to be determined.

Most of the substances thus far reported to activate complement by an antibody-independent C1 activation do so by virtue of their high surface density of negative charges such as phosphate or sulphate groups [30]. The structure of CL consists of four acyl chains, buried within the plane of the membrane, and two negatively charged phosphate groups, inserted between three derivatives of glycerol. These negatively charged phosphate groups are exposed on the outside of the membrane and may provide a possible site for C1q binding [15]. Our data suggest that other negatively charged liposomes such as PG and PI may, in fact, act in a similar manner. It is important to note that the amount of complement fragments such as iC3b deposited on cardiolipin-containing liposomes exceeded that found when PI was present but was comparable to liposomes containing PG. Thus, it is possible that the chemical structure of the anionic phospholipid may affect the amount of complement deposition. Furthermore, direct comparisons between the results of the hemolytic or C1q assays and immunoblot analysis must be made with caution. The former assays measure overall complement activation in the fluid phase of the reaction; the latter quantitates complement activation fragments that bind to the liposome surface – itself highly dependent on the availability of acceptor sites.

The dissection of the molecular interactions occurring between complement proteins and liposomes is most readily carried out in purified protein systems [15]. However, in the absence of other serum proteins, protein–lipid interactions may take place that are not seen in a whole serum system. Our model was selected to analyze complement activation in a system in which all other serum proteins were present. Thus, liposomes are able to interact with lipophilic proteins such as β-2-glycoprotein 1, a reputed cofactor protein for antiphospholipid antibodies [36,37]. It is possible that these cofactors or other serum proteins are also required for complement consumption in the absence or presence of immunoglobulins.

The incorporation of negatively charged phospholipids into liposomes reduces the circulation residency time in animal models [33,38]. It is likely that even neutral compositions acquire some amount of C3b on their surface as they circulate. The incorporation of derivatized phospholipids designed to modulate protein–phospholipid interactions into liposomes should reduce the complement-mediated clearance of liposomes from the circulation. Thus far, it has been shown that 'stealth' liposomes which contain polyethylene glycol (PEG)-derivatized phospholipids have prolonged circulation and stability in the presence of serum [39–41]. The polymer coat on these liposomes may be sufficient to prevent the binding of C1q in vivo. In both complement consumption and C1q binding assays, PEG-derivatized PE or CH was capable of blocking in

vitro complement activation by CL-liposomes (Bradley, Devine and Brooks, in preparation).

The pathway by which complement is activated in serum by liposomes has not been completely defined and may vary by experimental model. Large multilamellar vesicles (MLV) composed of hydrogenated egg PC, CH and diaceytl phosphate, which bear a net negative charge, have recently been shown to activate the alternative pathway in rat plasma [10]. The studies reported here cannot be directly compared to those of Funato et al. [10] owing to significant differences in liposome composition with respect to charge density, size, fluidity and phospholipids as well as the ratio of protein to liposomal lipid. We have found that liposome size, charge density and cholesterol content have a definite effect on complement consumption in rat serum [42]. In addition, our two studies differ in choice of species; complement activation has been shown to vary considerably among species [43]. Importantly, the studies of Funato et al. [10] were carried out in heparinized plasma which has different complement activity than heparin-free serum [44]. Our study confirms that, in human serum, no significant activation of the alternative pathway occurs [19], as no complement consumption was observed with any of the compositions used. Importantly, no rise in the levels of the activation fragment Bb was detected by ELISA, nor was any fragment Bb detected on the liposome surface using Western blot techniques. We did, however, detect iC3b on the surface of negatively charged liposomes that had been incubated in human serum. This observation suggests that we measured enzymatic complement activation in our system, not adsorption of complement factors onto the liposome surface. The extent of complement activation by liposomes remains to be determined. We failed to detect C7 bound to the liposome surface using immunoblotting methods and were able to visualize only a small amount of C9. Whether complement activation does not proceed efficiently past the C3b step on 100 nm vesicles or whether our detection system was insensitive remains to be determined.

Therefore, we have demonstrated that in an anticoagulant-free model system containing all serum proteins other than complement-activating immunoglobulins, liposomes bearing a net negative charge cause direct activation of the classical pathway of complement through the binding of C1, the first component of complement; naturally-occurring anti-phospholipid antibodies are not required.

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