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Liposome–complement interactions in rat serum: implications for liposome survival studies

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Abstract

Serum complement opsonizes particles such as bacteria for clearance by the reticuloendothelial system. Complement has been reported to interact with liposomes and therefore may mediate the reticuloendothelial system clearance of liposomes. This study has used a rat serum model to define some of the characteristics of liposomes which modulate their ability to activate complement. Using functional hemolytic assays and C3/C3b crossed immunoelectrophoresis, we have demonstrated that liposomes activated rat complement in a dose-dependent manner with higher concentrations of liposomes activating higher levels of complement. The detection of complement activation required the inclusion of phospholipids bearing a net charge. Complement activation occurred via the classical pathway; no alternative pathway activation was detected. The presence of cholesterol contributed to complement activation in a dose-dependent manner. Phospholipid fatty acyl chain length did not influence complement activation while the introduction of unsaturated acyl chains markedly decreased levels of complement activation. Liposome size also influenced complement activation with 400 nm unilamellar vesicles more effectively activating complement than 50 nm vesicles for equivalent amounts of exposed lipid. These studies demonstrate that the composition of the liposome greatly affects the in vitro activation of rat serum complement and suggest that the biological half-life of liposomes in the circulation of rats may be altered by changing the liposome composition to reduce complement activation.

Key words: Liposome; Complement; Blood protein; Drug carrier

1. Introduction

Deposition of complement onto a cell membrane marks it for immune clearance. Liposomes provide a model membrane system in which to study the physicochemical characteristics that influence the degree to which complement is activated. Depending on their lipid composition and cholesterol content, liposomes can activate the complement system of several species including human, guinea pig and rat [1,2]. The opsonization of liposomes by complement has practical importance in that liposomes are used clinically for targetted drug delivery. In vivo studies in animal models have shown that the principal clearance sites for liposomes are liver and spleen, that is, the reticuloendothelial system [3]. It is likely that complement plays a role in opsonizing liposomes for clearance.

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Abbreviations: MLV, large, multilamellar vesicle; SUV, small, unilamellar vesicle; PI, phosphatidylinositol derived from bovine liver; PC, phosphatidylcholine derived from egg; PG, phosphatidylglycerol derived from egg; CL, cardiolipin; CHOL, cholesterol; SA, stearylamine; DOTAP, 1,2-bis(oleoloxy)-3-(trimetholammonio)propane; PA, phosphatidic acid from egg; DMPC, dimyristoylphosphatidylcholine, a 14:0/14:0 PC; DMPG, dimyristoylphosphatidylglycerol, a 14:0/14:0 PG; DOPC, dioleioylphosphatidylcholine, a 18:1/18:1 PC; DOPG, dioleioylphosphatidylglycerol, a 18:1/18:1 PG; DPPC, dipalmitoylphosphatidylcholine, a 16:0/16:0 PC; DPPE, dipalmitoylphosphatidylethanolamine, a 16:0/16:0 PE; DPPG, dipalmitoylphosphatidylglycerol, a 16:/16:0 PG; DSPC, distearoylphosphatidylcholine, a 18:0/18:0 PC; DSPG, distearoylphosphatidylglycerol, a 18:0/18:0 PG; EA, sheep erythrocytes coated with antibody; EAC1,4,2: sheep erythrocytes bearing C4b2a complexes; EGTA, ethylene glycol-bis(oxyethylenenitrilo)tetraacetic acid; VBS, veronal-buffered saline; GVB, gelatin veronal-buffered saline; DGVB, dextrose, gelatin, veronal-buffered saline.

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The rat model is frequently employed to study the in vivo behavior of liposomes. Although several in vitro studies of the parameters affecting complement activation in human and guinea pig serum have been reported [4–7], no information is available concerning the complement activating properties of liposomes in rat serum. This is not insignificant since species differences in complement function are well known [8] and the in vivo behavior of opsonized liposomes is different among animal models. Several factors have been identified that influence the in vivo survival of liposomes. These include the charge of the lipid used in liposome composition and liposome size. In the rat model, liposomes bearing a net positive or neutral charge remain in the circulation longer than liposomes bearing a negative charge [9,10]. The size of a liposome greatly influences its survival. Juliano and Stamp [9] demonstrated that small unilamellar vesicles (SUV) are less rapidly cleared than large multilamellar vesicles (MLV). Liposome size also influences the degree of immune-mediated damage. In the presence of specific antiphospholipid antibody, MLV are more resistant to complement-mediated lysis in human serum than SUV; however, within a population of MLV, the smaller ones are more susceptible to complement damage [4]. Paradoxically, large unilamellar vesicles are more resistant to complement-mediated lysis than MLV. We have undertaken studies to identify the physicochemical characteristics that affect complement activation in rat serum. In addition, we have investigated the effect of liposome size on the complement activating ability of large unilamellar vesicles prepared by extrusion techniques.

2. Materials and methods

Reagents

Rat serum complement was purchased from Accurate Chemical, Westbury, NY. Antiserum to sheep RBC and functionally purified guinea pig C1, human C4 and guinea pig C2 were obtained from Diamedix, Miami, FL. The 1,2-bis(oleoloxy)-3-(trimetholammonio)propane (DOTAP) used in these studies was the gift of Dr. J.R. Silvius, McGill University, Montreal. Cholesterol (CHOL) and stearylamine (SA) were obtained from Sigma, St. Louis, MO. All other lipids used in these studies were purchased from Avanti Polar Lipids, Pelham, AL. These included phosphatidylcholine derived from egg (PC), phosphatidylglycerol from egg (PG), phosphatidic acid from egg (PA), phosphatidylinositol from bovine liver (PI), phosphatidylserine from bovine brain (PS), cardiolipin from bovine heart (CL), dimyristoylphosphatidylcholine, a 14:0/14:0 PC (DMPC), dimyristoylphosphatidylglycerol, a 14:0/14:0 PG (DMPG), dioleioylphosphatidylcholine, a 18:1/18:1 PC (DOPC), dioleioylphosphatidylglycerol, a 18:1/18:1 PG (DOPG), dipalmitoylphosphatidylcholine, a 16:0/16:0 PC (DPPC), dipalmitoylphosphatidylethanolamine, a 16:0/16:0 PE (DPPE), dipalmitoylphosphatidylglycerol, a 16:0/16:0 PG (DPPG), distearoylphosphatidylcholine, a 18:0/18:0 PC (DSPC) and distearoylphosphatidylglycerol, a 18:0/18:0 PG (DSPG).

Preparation and characterization of liposomes

The standard methods for the preparation of liposomes used in these studies are described in detail elsewhere [11,12]. Large unilamellar vesicles in veronal-buffered saline (150 mM NaCl, 75 mM sodium barbital (pH 7.5)) (VBS) were prepared by extruding a lipid mixture under nitrogen pressure below 500 psi through two 25 mm polycarbonate filters placed in a liposome extrusion apparatus (Lipex Biomembranes, Vancouver, B.C.). To produce different sized vesicles, filter pore size was varied from 50 nm to 400 nm. All vesicles were extruded a minimum of ten times, then sized using quasi-elastic light scatter data subjected to NICOMP (model 270) analysis using a vesicle Gaussian unimodal distribution mode. Preparations were performed at room temperature except for the extrusion of saturated phospholipids or those involving 50 nm pore size filters where heated barrel chambers and higher pressures were required. The liposomes were suspended in VBS containing 0.1% dextrose, 0.1% gelatin, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ ($DGVB^{2+}$) for assays. For some studies, liposomes were also characterized by phosphorous NMR to determine the percentage of phospholipid on the liposome surface. Determinations of exposed phospholipid were calculated based on the quenching by Mn^{2+} of the ^{31}P-NMR signal arising from phospholipids in the exterior monolayer employing a Barker MSL 200 apparatus [12].

Complement-liposome incubation and hemolytic assay

The functional interaction between complement and liposomes was measured in a two-stage assay. In the first stage 100 μl of liposome suspension was incubated for 30 min at 37°C with 100 μl of rat serum diluted 1:3 in $DGVB^{2+}$. After incubation, 300 μl of ice-cold $DGVB^{2+}$ were added. In the second stage, a hemolytic assay was used to determine the remaining complement activity. For most experiments, antibody-coated sheep red blood cells (EA) were used as a target for complement-mediated lysis. Sheep red blood cells were suspended to a concentration of $1 \cdot 10^9$ cells/ml in veronal-buffered saline containing 0.1% gelatin and 20 mM EDTA (EDTA-GVB) and incubated with an equal volume of a 1:500 dilution of IgG anti-sheep red cell hemolysin for 30 min at 37°C. EA were washed three times in $DGVB^{2+}$ to remove unbound protein and

replenish divalent cations, then suspended to a final concentration of $1.5 \cdot 10^8$/ml. 50 μl of EA were then incubated for 30 min at 37°C with 50 μl of serum previously exposed to liposomes. 2 ml of ice-cold EDTA-GVB was added to inhibit any further cytolysis. Unlysed EA were removed by centrifugation and the percentage of cells lysed determined by measuring hemoglobin release at a wavelength of 414 nm. Lysis of the target cell is directly correlated with the level of residual active complement in the serum after exposure to liposomes. A lowered level of active complement after liposome exposure is interpreted as evidence of complement consumption during the incubation of rat serum and liposomes.

Preliminary studies indicated that optimum sensitivity of the hemolytic assay for the detection of complement activation in serum exposed to liposomes required a final dilution of the serum to between 1:15 and 1:25. This dilution was performed in two steps in order to prevent dilution of serum below the amount required to maintain active complement in the incubation with liposomes. Therefore, rat serum was diluted 1:3 prior to incubation with liposomes and then 1:5 prior to the hemolytic assay, achieving a final dilution of 1:15.

Determination of the pathway of complement activation

The divalent cation requirements for complement activation differ for the classical and alternative pathways; classical pathway requires calcium and magnesium ions while the alternative pathway requires only magnesium ions [13]. In order to measure activation by the alternative pathway, liposomes were incubated with Ca^{2+}-depleted rat serum to prevent classical pathway activation. Ca^{2+}-depleted rat serum was prepared by incubating 9 parts rat serum with 1 part 0.2 M EGTA for 5 min at 37°C. The serum was then made 10 mM with respect to $MgCl_2$ and diluted to a final dilution of 1:2 with $DGVB^+$ ($DGVB^{2+}$ minus Ca^{2+}) prior to the addition of liposomes. The consumption of C3–C9 was assessed by incubating these serum samples with sheep erythrocytes bearing C4b2a complexes (EAC1,4,2). EAC1,4,2 were prepared by incubating EA at $1 \cdot 10^9$ in $DGVB^{2+}$ with guinea pig C1 (5000 U/ml of EA) for 5 min, human C4 (5000 U/ml EA) for 10 min and guinea pig C2 (250 U/ml EA) for 7 min at 37°C. The EAC1,4,2 were then cooled in an ice bath, washed three times at 4°C in $DGVB^+$ and resuspended to $1.5 \cdot 10^8$/ml. $DGVB^+$ was used in the serial liposome dilutions as well. The ability of the EAC1,4,2 assay to detect consumption of C3–C9 was determined using serum treated with inulin, an alternative pathway activator. Preliminary studies demonstrated that rat serum can be diluted to 1:8 without decreasing its hemolytic capacity in this assay (data not shown).

Crossed immunoelectrophoresis

To directly identify the conversion of native rat C3 to activated rat C3b in serum exposed to complement activating and non-activating liposomes, crossed immunoelectrophoresis was performed. Rat serum was diluted 1:4 in VBS, then incubated for 30 min at 37°C with 10 mM PC/PG liposomes, non-activating composition, at a mole ratio of 80:20, or 10 mM PC/CHOL/PG at 35:45:20, an activating composition. Serum exposed to zymosan was used as a positive control and untreated serum was also tested. Liposomes and zymosan were removed from serum samples by centrifugation at $12000 \times g$ for 10 min. For first dimension electrophoresis, all four samples were run in the same gel at 10 V/cm. After completion of electrophoresis, each sample strip was sliced from the gel and electrophoresed at a right angle to the first electrophoresis at 2 V/cm overnight into a 1% agarose gel containing goat anti-rat C3 (Organon Teknika, Durham, NC). The gel was then soaked in saline to remove unprecipitated proteins, dried and stained with Coomassie blue R-250. In CIE, the first dimension electrophoresis separates the proteins by size and C3b migrates further than C3. For these experiments, all first dimension electrophoreses were carried out in the same gel to allow direct comparison among samples.

All experiments described above and in the results section were repeated a minimum of three times.

3. Results

The effect of exposure to liposomes on the functional complement activity in serum was measured using a hemolytic assay in which serum was reacted with antibody-sensitized sheep red cells. When no lipid was present, 100% lysis of target erythrocytes occurred indicating no consumption of complement. However, at high concentrations of some lipids, low levels of lysis of target erythrocytes occurred indicating consumption of complement during exposure to liposomes. The level of complement activation was dependent on the amount of liposomes added to the serum; lipid concentration and residual functional complement were inversely proportional (Fig. 1). Liposomes with a net positive (PC/CHOL/SA or PC/CHOL/DOTAP) (Fig. 1, top panel) or negative charge (PC/CHOL/PG, PC/CHOL/PA, PC/CHOL/CL, PC/CHOL/PI or PC/CHOL/PS) (Fig. 1, bottom panel) consumed complement at lipid concentrations exceeding 1 mM whereas neutral liposomes did not activate complement at any concentration tested (Fig. 1, center panel).

In order to assess alternative pathway activity, calcium-depleted serum was exposed to either positively charged (PC/CHOL/SA), negatively charged (PC/CHOL/PA and PC/CHOL/PG) or neutral (PC/

CHOL) liposomes. The depletion of serum calcium prior to incubation with liposomes blocks classical pathway activation at C1. The activation of complement in calcium-depleted but not magnesium-depleted serum is a distinguishing characteristic of alternative pathway activation, residual C3–C9 activity was measured by

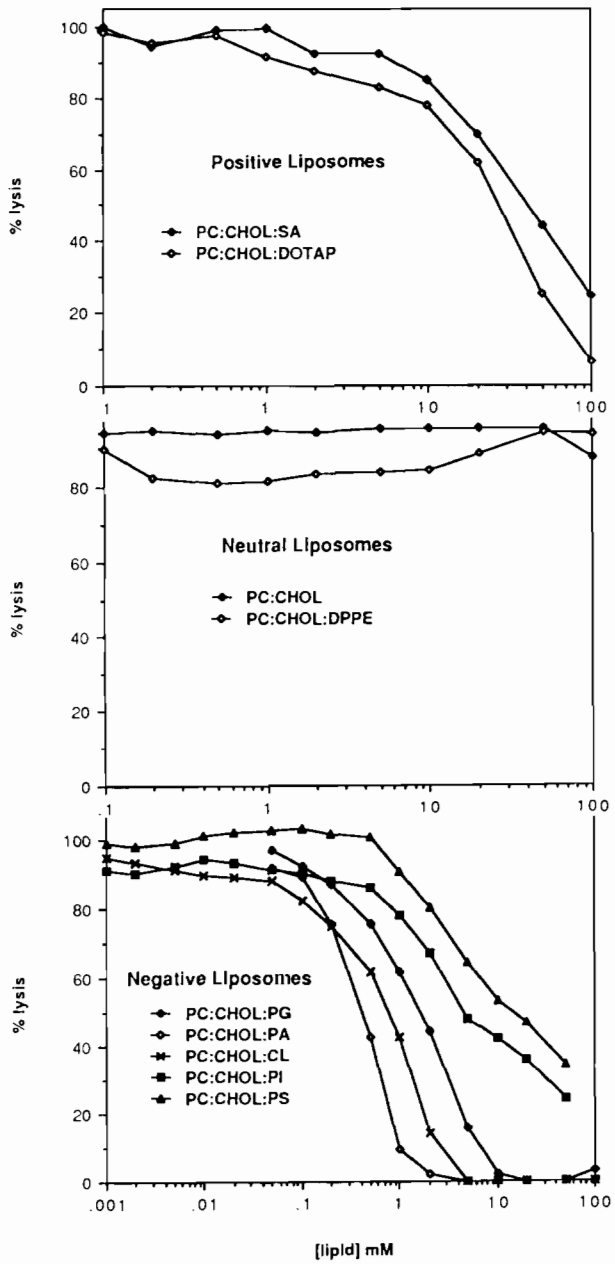

Fig. 1. Complement consumption as a function of liposomal charge and concentration. 100 nm liposomes were prepared using lipids that were positively charged (top panel), neutral (middle panel) or negatively charged (bottom panel) at molar ratios of 55:45 for PC/CHOL and 35:45:20 for PC/CHOL/lipid indicated. Liposomes were diluted serially in $DGVB^{2+}$ and incubated for 30 min at 37°C in normal rat serum diluted 1:4. Residual complement activity, expressed as % lysis of target cells, was measured by hemolysis of antibody-sensitized erythrocytes.

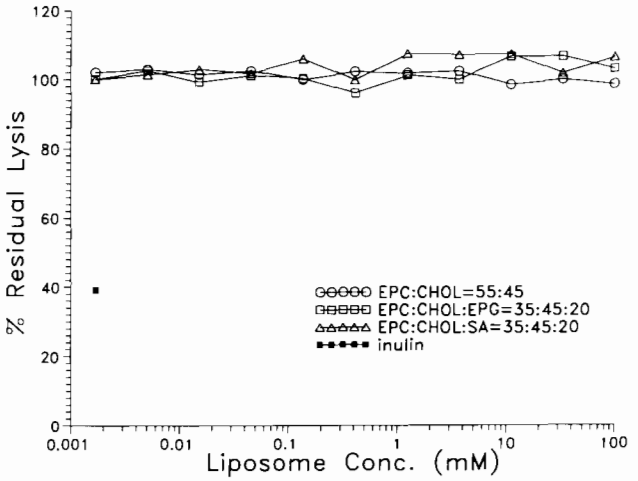

Fig. 2. Complement activation by liposomes under conditions inhibiting the classical pathway. One hundred nm liposomes were incubated in rat serum diluted in $DGVB^{2+}$ or EGTA-DGVB supplemented with Mg^{2+} at a final concentration of 1:2 for 30 min at 37°C. Liposomes were made with a molar ratio of 55:45 for PC/CHOL and 35:45:20 for PC/CHOL/lipid indicated. As a positive control for the ability of the assay to detect alternative pathway activation, rat serum diluted as above was incubated with 10 mg/ml inulin. Residual complement activity of C3–C9 was measured using erythrocytes bearing C4b2a (EAC1,4,2).

incubating the liposome-treated serum samples with sheep erythrocytes bearing pre-formed C4b2a (EAC1, 4,2) Calcium-depleted serum exposed to any liposome compostition demonstrated no change in hemolytic activity relative to buffer controls; however, the assay readily detected activation of the alternative pathway by the inulin control (Fig. 2). These experiments suggest that liposomes do not activate rat complement by the alternative pathway at a detectable level.

To assess the effect of cholesterol on classical pathway complement activation, PC/PG liposomes were prepared containing from 0 to 45 mol% cholesterol and a constant PC/PG ratio of 2:1. When these liposomes were exposed to serum, complement consumption increased in direct porportion to the amount of cholesterol included in the liposome composition (Fig. 3).

Liposomes of similar charge and cholesterol content but varying saturation and fatty acyl chain length were prepared and tested for their ability to activate complement. Fatty acyl chain length or saturation were changed in net neutral liposomes by using DPPC, DSPC, DOPC or DMPC in place of PC. No complement activation was detected in serum incubated in liposomes made of these modified PC liposomes with or without the addition of 45 mol% cholesterol (data not shown). Different results were obtained when the saturation or fatty acyl chain length were altered in liposomes carrying a net charge. Phospholipid chain

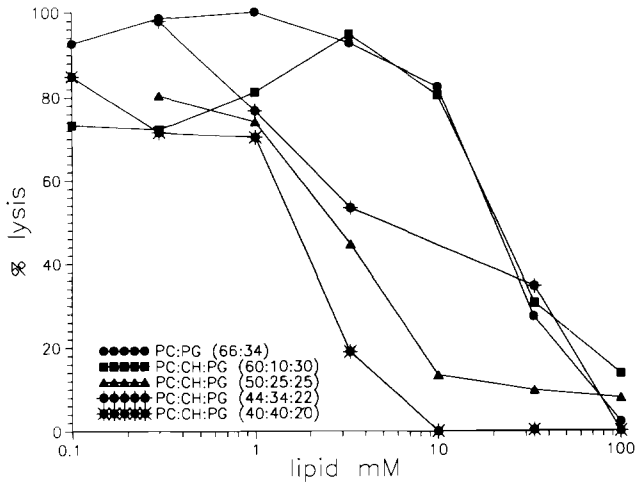

Fig. 3. The effect of liposome cholesterol content on complement activation. 100 nm liposomes (PC/CHOL/PG) containing increasing concentrations of cholesterol and a constant ratio of PC/PG were incubated for 30 min at 37°C with rat serum diluted 1:4 in GVB^{2+}. Residual complement activity was assessed by incubating rat serum with antibody-sensitized erythrocytes.

length was changed in complement activating liposomes by preparing liposomes composed of DPPC/CHOL/DPPG, DSPC/CHOL/DSPG or DMPC/CHOL/DMPG, all with molar ratios of 35:45:20. At the lowest lipid concentrations tested, the activation of complement was more efficient with shorter fatty acyl chain length; liposomes containing DMPC activated more complement (42% residual activity) than liposomes containing DPPC (63% residual activity) which activated more complement than liposomes containing DSPC (77% residual activity) (Fig. 4A). Complement activation was maximal at lipid concentrations $\geq$ 1 mM. The inclusion of unsaturated phospholipids such as DOPC and/or as the activator determinant, DOPG, markedly decreased the capacity of the membrane to activate complement (Fig. 4B). Unlike saturated systems, complete lysis of the EA targets occurred at lipid concentrations up to 1 mM indicating no complement activation by the liposomes was detected. More than 20 times the amount of lipid was required for complement activation if phospholipids were unsaturated.

The influence of vesicle size on complement activation is of interest because vesicle size has been shown to influence in vivo clearance behavior; large vesicles are cleared more rapidly than small systems [9]. Thus, the complement activating capacities of PC/CHOL/PG liposomes (35:45:20) sized through filters with 50, 100, 200, or 400 nm pore sizes were examined. As shown in Fig. 5A, the smaller vesicles were somewhat less effective at activating complement (for equivalent concentrations) as determined by the consumption assay. However, as shown elsewhere [14], at a given lipid concentration, the larger vesicles exhibit less exposed surface area than the smaller unilamellar vesicles due to the multilamellar character of larger systems. In order to determine the amount of exposed lipid, ^{31}P-NMR studies were performed employing the ability of manganese to quench the ^{31}P-NMR signal of exterior phospholipids. As indicated in Table 1, at the same lipid concentration, the smaller vesicles exhibit considerably more exposed surface area. After correcting for this factor, the smaller vesicles were observed to be much less effective complement activators than larger vesicles (Fig. 5B).

Because the hemolytic assay employed for most of these studies measures the amount of functional complement remaining in the sample, it was necessary to demonstrate that a reduction in hemolytic activity could be attributed to the conversion of C3 and not to

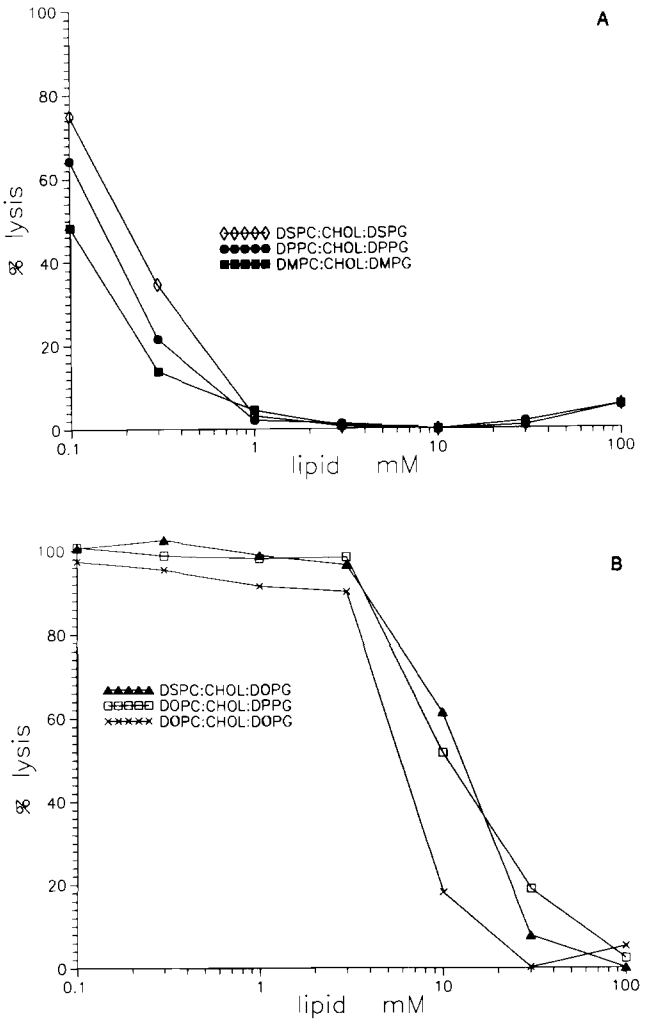

Fig. 4. The effect of fatty acyl chain length and saturation on complement activation. The residual complement activity after incubation in rat serum diluted 1:4 in GVB^{2+} for 30 min at 37°C is shown for 100 nm liposomes prepared using lipids of differing fatty acyl chain length (A) or with unsaturated lipids included (B). Residual complement activity was measured using antibody-coated sheep erythrocytes.

Table 1
Size characteristics of PC/CHOL/PG (35:45:20) liposomes prepared by extrusion

Filter pore size (nm)	Mean diameter QELS (nm)	Exposed lipid (%)
50	69	58
100	103	51
200	164	41
400	250	35

non-specific absorption to the liposomes. To measure the enzymatic activation of C3, we performed crossed immunoelectrophoresis analysis of rat serum samples exposed to complement activating (PC/CHOL/PG) and non-activating liposomes (PC/PG) as well as untreated and zymosan-treated specimens. The cleavage of a cationic 9 kDa fragment from the alpha chain of C3 by the C3 convertase enzymes results in a shift in the electrophoretic mobility of the resultant fragment, C3b, relative to native C3 [15]. For samples exposed to liposomes, the distance of the peak of the precipitin arc from the origin was compared to serum samples which were either untreated, and therefore contain mostly C3, or exposed to the potent complement activator zymosan, and therefore contain mostly C3b. As can be seen in Fig. 6, the migration distance from the origin for the native C3 molecule was 2.90 cm (panel D); in zymosan-treated serum, this distance increased to 3.90 cm (panel C). Serum incubated with 10 mM PC/PG liposomes had the major precipitin arc at 2.90

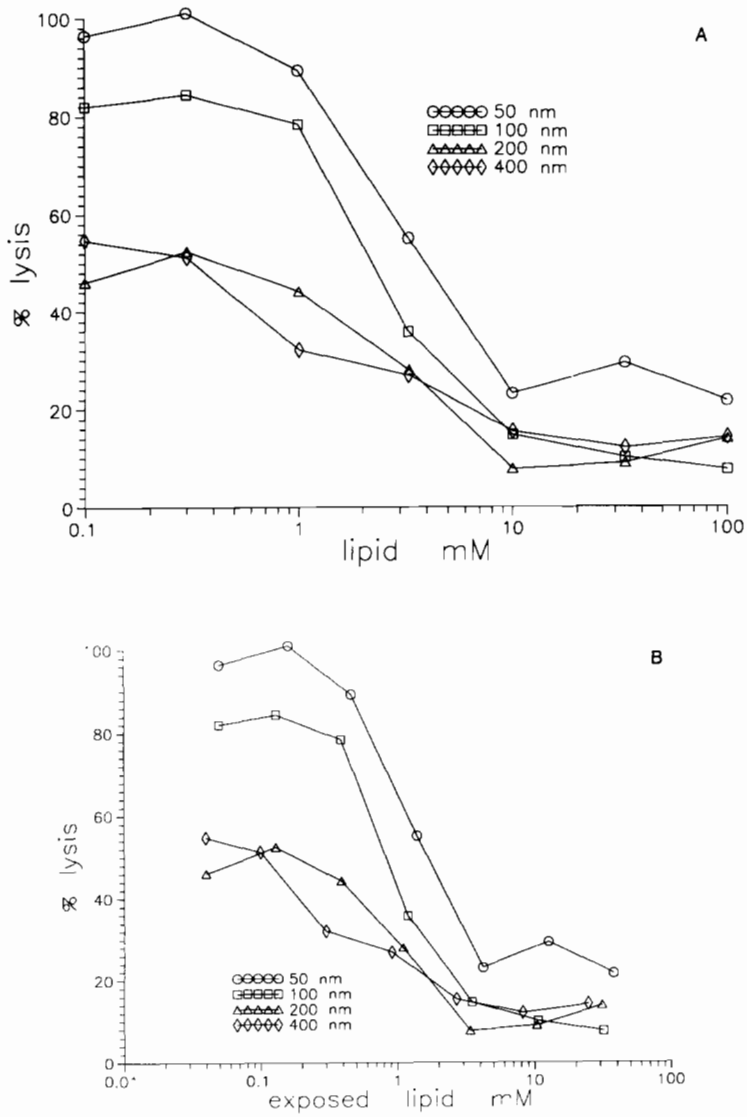

Fig. 5. The effect of liposome size on complement activation. Liposomes of 35:45:20 molar ratio of PC/CHOL/PG were extruded through filters of the sizes indicated. After incubation for 30 min at 37°C in rat serum diluted 1:4 in GVB^{2+}, the residual hemolytic complement activity was assessed using antibody-sensitized sheep erythrocytes. In panel A, data are expressed as moles of lipid added, while the data shown in panel B have been corrected for the percentage of lipid exposed as determined from ^{31}P-NMR studies of each liposome preparation tested.

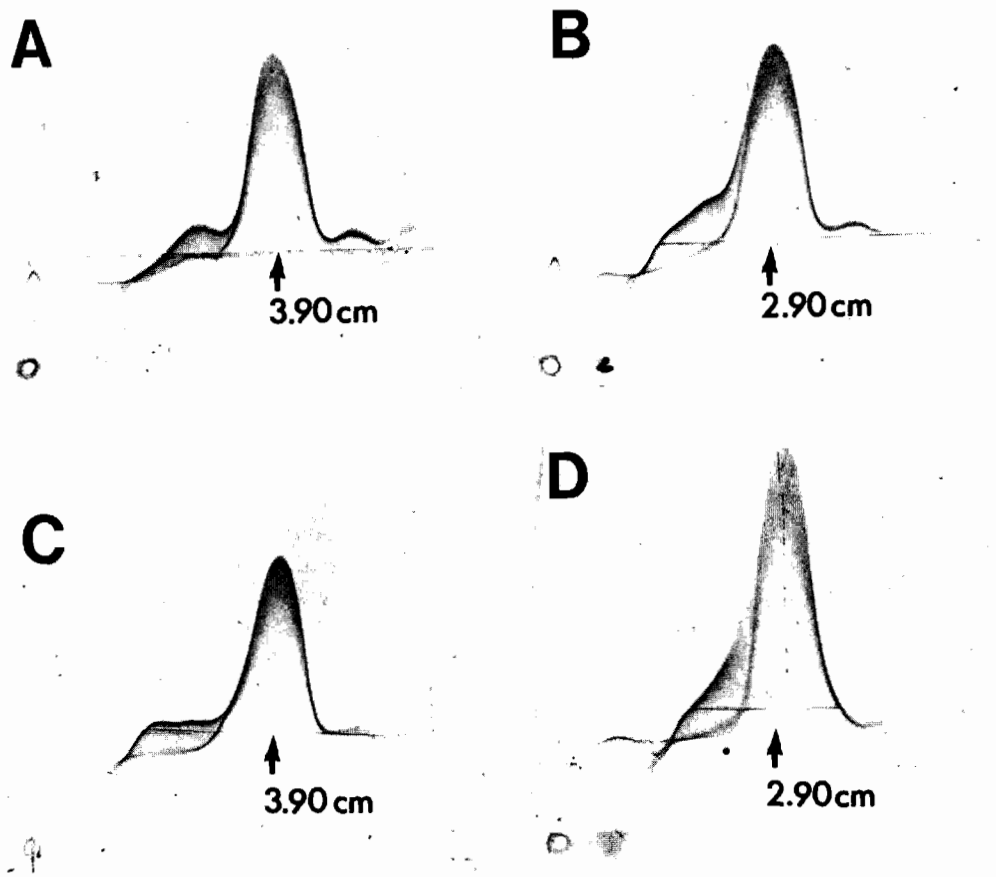

Fig. 6. Two-dimensional crossed immunoelectrophoresis of rat C3/C3b in serum exposed to (A) PC/CHOL/PG (35:45:20) liposomes, (B) PC/PG (80:20) liposomes, (C) zymosan, or (D) $DGVB^{2+}$. Serum samples were electrophoresed in the second dimension into an agarose gel containing goat anti-rat C3. The precipitin peak for native C3 migrated at 2.90 cm from the origin while C3b migrated 3.90 cm from the origin.

cm (panel B) indicating no conversion of C3 to C3b. Serum exposed to PC/CHOL/PG (35:45:20) liposomes formed a major precipitin arc at 3.90 cm (panel A) indicating conversion from C3 to C3b. These results directly parallel those described above for functional studies of the effect of cholesterol on complement activation (Fig. 3) in which PC/PG liposomes failed to consume complement while PC/PG liposomes containing 45 mol% cholesterol showed marked consumption of complement.

4. Discussion

The studies reported here have identified some of the parameters that influence the interactions of liposomes with the proteins of the rat complement system. The addition of lipids bearing a net charge to the liposome resulted in the activation of complement; charged liposomes activated complement in a dose-dependent manner. Net neutral liposomes failed to activate complement under these experimental conditions. The importance of surface charge in complement activation is not unique to charged lipids. Other studies have clearly demonstrated that the surface charge of bacteria or red blood cells markedly influences the level of complement activation [16,17]. Surface charge density may also affect the level of complement activation since liposomes containing lipids with the greatest net charge on the head group activated the most complement. The effect of charge on complement activation in serum is paralleled by the effect of charge on in vivo clearance of liposomes; Juliano and Stamp demonstrated that negatively charged liposomes are cleared from the circulation of rats more rapidly than uncharged or positively charged liposomes [9]. Recent work by members of the present collaboration has demonstrated that the amount of protein bound to the surface of liposomes after circulation in CD1 mice is inversely proportional to the circulation residency time [10]. Those compositions that were rapidly cleared from the circulation, including CL- and PA-containing liposomes, are the same compositions that demonstrated the strongest degree of complement activation in this study. The liposomes used in both studies were the same size and had the same cholesterol content. Similarly, liposomes that circulated for a moderate length of time, for example, those containing PI, were less

complement activating than CL-containing compositions in the present study. Thus, there is a direct correlation between the complement activating ability of liposomes and their circulation residency time in vivo.

The activation of rat complement by charged liposomes proceeded only via the classical pathway. Irrespective of the lipid used to prepare the liposomes, we were unable to demonstrate measurable activation of the alternative pathway in liposome-treated serum. This observation highlights the species differences in the complement system [8] since these results differ from those reported by our group and others for liposome-treated human serum [18,19]. In the human system, complement activation can proceed via the alternative pathway if the lipid has a net positive charge and via the classical pathway if the lipid has a net negative charge [19]. Recently, Funato et al. demonstrated the activation of the alternative pathway of complement in rat plasma by PC/CH liposomes containing dicetyl phosphate [20]. Although the system employed by these investigators differs in several respects from the one reported herein, it is likely that the use of heparinized plasma as a source of complement in the Funato et al study is responsible for the discrepancy in results. Polyanions, including the anticoagulant heparin sulfate, are known to accelerate the activation of the alternative pathway by directly binding factor H [21]. The loss of this regulatory protein of the alternative pathway is measured as alternative pathway activation. Furthermore, we have demonstrated that in heparin-free human serum systems, carboxyfluorescein is not released by exposure of PC/CH liposomes to normal human serum or serum deficient in C1q [19]. In our system, which is free of added heparin, we found no alternative pathway activation in rat serum induced by either negatively or positively charged phospholipids.

The studies reported here indicate that increasing the cholesterol content of the liposome increases the degree of rat complement activation. This observation is consistent with earlier findings from guinea pig [7,22] and human studies [19]. The mechanism by which cholesterol facilitates complement fixation is unclear. The effect may be direct; crystalline cholesterol is an activator of human complement in vitro [23] and complement is present in atherosclerotic lesions [24]. In addition, alternative pathway activation in human serum requires cholesterol to mediate the binding of serum factors to target molecules [25,26]. Whether the presence of cholesterol could enhance complement activation by altering the physical properties in the hydrocarbon region of the bilayer is debatable. The ability of cholesterol to increase the interactions of antibodies and complement with membranes has been attributed by other workers to a decrease in membrane 'fluidity' [22]. This is consistent with the fact that liposomes containing unsaturated lipids are much less effective complement activators than those containing saturated lipids with the same acyl chain length (Fig. 4B). However, lipids containing longer saturated acyl chains exhibit increased acyl chain order or decreased fluidity compared to those containing shorter acyl chains [27]. Thus, if increased membrane order leads to an increased ability to activate complement as may be suggested by the results for cholesterol, similar effects should be observed as the acyl chain length is increased. This is not the case (see Fig. 4A). Studies of human complement suggest that the complement activation associated with decreasing chain length results not from increasing membrane fluidity but the masking of binding sites in the bilayer when longer phospholipids are used [5,19,28].

Although charge and cholesterol content influenced complement activation to a greater degree than fatty acyl chain length, unsaturation of the acyl chains significantly reduced complement activation. The presence of unsaturated lipid resulted in a 20-fold higher lipid concentration to achieve the same degree of complement activation as a saturated system. This observation is in agreement with findings on human complement [19] as well as guinea pig complement [28] where the introduction of more than one unsaturated bond decreases complement activation. Changes in unsaturation may affect the competition between phospholipid-phospholipid interactions and complement protein-phospholipid interactions. Studies of Shin et al. [28] suggest that more favorable complement protein-phospholipid interactions may arise from exposure of the phospholipid binding site for complement factors such as C4.

In vivo studies in rats have shown that larger vesicles are cleared from the system at a much faster rate than smaller vesicles [9]. Although a role for complement in this clearance has yet to be demonstrated conclusively, different efficiencies of complement activation for different sized vesicles could contribute to the observed clearance rates. We have demonstrated that for a given amount of lipid exposed to serum, liposome size was directly proportional to the level of rat complement activation. This observation was unexpected because at a fixed lipid concentration, the total surface area and number of theoretic binding sites exposed is greatest in the smallest liposomes. The marked reduction of complement activation by 50 and 100 nm liposomes may be due to the requirements for the assembly and activation of complement proteins. Local geometry and surface dynamics markedly affect the activation of the initial proteins of the complement cascade. For example, it has been postulated that the activation of C1 requires the multivalent binding of multiple C1q globular regions within the C1qrs complex [29] and C2 activation by C1s is dependent on the geometry of the

local deposition of C4b. On the more curved surfaces of smaller liposomes, the proper geometric configuration for efficient complement activation may be achieved less readily than on larger liposomes. The studies reported herein identify a set of physicochemical characteristics of liposome-complement protein interactions that can be used in the formulation of liposomes that are complement activating or complement non-activating in rat serum. These parameters can be used to develop strategies for the design of liposomes that have either shortened or prolonged survival in rat models of liposomal delivery systems.

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