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THE ROLE OF SURFACE CHARGE IN THE ACTIVATION OF THE CLASSICAL AND ALTERNATIVE PATHWAYS OF COMPLEMENT BY LIPOSOMES[1]

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We have studied the complement-activating properties of liposomes. We show that surface charge is a key determinant of complement-activating liposomes. The nature of the charge, whether negative or positive, appears to dictate which pathway of the complement system is activated. Phosphatidylcholine:cholesterol (PC:CHOL, 55:45 mol/mol) liposomes were made to exhibit a positive or negative surface charge by the addition of cationic or anionic lipids, respectively. Normal human or guinea pig serum was incubated with liposomes, followed by determining the residual hemolytic activity of the serum as a measure of complement activation. Negatively charged liposomes containing phosphatidylglycerol, phosphatidic acid, cardiolipin, phosphatidylinositol, or phosphatidylserine activated complement in a Ca^{2+}-dependent manner suggesting activation occurred via the classical pathway. Positively charged liposomes containing stearylamine or 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane activated complement via the alternative pathway. Neutral liposomes, PC:CHOL (55:45) and PC:CHOL: dipalmitoylphosphatidylethanolamine (35:45:20), failed to activate complement as measured by the hemolytic assays. We show that unsaturated liposomes are more potent complement activators than saturated liposomes and that 45 mol% cholesterol promotes complement protein-liposome interactions. Immunoblot analysis of phosphatidylglycerol-containing liposomes showed that C3b and C9 were associated with these liposomes. Thus, the complement consumption measured in the hemolytic assays represents active cleavage of the complement components and not passive adsorption to the liposome surface. These studies suggest that membranes composed of net charged phospholipids can activate the complement system. This observation underlines the importance in biologic membranes of complement regulatory proteins that protect normal cells from complement attack.

Complement activation by the alternative pathway occurs when C3 interacts with particulate material such as bacterial cell wall constituents, immune complexes, or heterologous erythrocytes (1). The C3b, the activated form of C3, which is bound to particulate material has two possible fates. It may interact with factor B to produce a serine protease that amplifies the activation. Alternatively, the C3b may be bound by factor H that acts to stop the cascade by providing cofactor activity for the C3b inactivating protease, factor I (2). The characteristics of the surface that promote the binding of one factor over the other are not well understood. Studies of both bacteria and mammalian cells have suggested that sialic acid is an important regulator of complement activation (3, 4). Complement-resistant E (5) and the complement-resistant strains of *Escherichia coli* (6, 7) both contain more sialic acid residues than their complement-sensitive counterparts. These charged residues are thought to influence the binding affinities of factor B and factor H (8).

Complement activation by the classical pathway occurs when the first component of complement, C1, binds either to Ig or directly to a particle surface. The antibody-independent activation of C1 has been described for a wide variety of substances including DNA and RNA (9), urate crystals (10), Gram-negative bacteria (11), retroviruses (12), and heparin-protamine (13). The binding of C1 via the C1q moiety requires the presence of repeating binding sites to bind the multiple heads of the C1q molecule (14). The other factor identified to be important in binding C1q is a negative surface charge (15, 16).

Liposomes can be used as simple model membranes to study the influence of lipid composition on complement activation. This approach has been used to demonstrate the inhibitory effects of sialic acid on complement activation (17). Liposomes containing gangliosides or glycophorin are relatively inefficient complement activators whereas liposomes containing asialoglycolipids or neuraminidase-treated glycophorin fail to inhibit complement activation (18, 19). Recent studies using liposomes have demonstrated that sialic acid acts to promote the binding of factor H to C3b (17).

The interaction of complement proteins with liposomes also has important consequences for the use of liposomes as drug delivery systems. The deposition of activated complement proteins, especially those that function as opsonins, on the liposomal surfaces may contribute significantly to liposome clearance from the circulation, a key problem associated with liposomal drug delivery systems. This contribution is suggested by the recent findings of Allen and Chonn (20) that liposomes containing sialic acid in the form of gangliosides have significantly extended circulation times. An understanding of the complement-activating properties of liposomes, therefore,

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may allow the development of liposomal systems which have long biologic half-lives.

To date, the activation of the complement system by liposomes has been described for only a few specific lipid compositions. These compositions include liposomes containing haptenated lipids (21), phosphatidylserine with phosphatidylethanolamine (22), cardiolipin (16), stearylamine in the presence of galactosyl ceramide (23), cerebrosides in dimyristoylphosphatidylethanolamine (17), or saturated phosphatidylethanolamine in saturated phosphatidylcholine liposomes containing cholesterol (24). From reviewing these studies, no fundamental property of complement-activating liposomes could be readily defined because of inconsistencies in the reports and because of the complex systems studied.

We have extended these studies to demonstrate that surface charge is an essential component of complement-activating liposomes in human and guinea pig whole serum systems. We show that negatively charged liposomes activate complement via the classical pathway, that positively charged liposomes activate complement via the alternative pathway and that neutral liposomes are very weak activators. We also show that complement-activating liposomes have C3b associated with their membranes. Inasmuch as C3b functions as an opsonin, the coating of the liposomes with C3b may be responsible for the recognition of liposomes by the immune system as foreign particles.

MATERIALS AND METHODS

Preparation of liposomes. Multilamellar and FMLV[3] vesicles were prepared according to established methods (25). Large unilamellar vesicles were prepared by extrusion of multilamellar vesicles through polycarbonate filters of defined pore sizes (Nuclepore, Pleasanton, CA) using an extrusion device (Lipex Biomembranes, Vancouver, Canada) as previously described (25, 26). Lipids were purchased from the following companies: PC, PA, PG, PI, PS, CL, and dipalmitoylphosphatidylethanolamine (DPPE) from Avanti Polar Lipids, Pelham, AL; SA and CHOL from Sigma; 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP) was a generous gift from Dr. J. R. Silvius (McGill University, Montreal, Canada). These lipids were used without further purification. The standard liposome preparations used in the functional complement activation studies consisted of PC:CHOL (55:45 mol/mol) large unilamellar vesicles extruded through 100 nm polycarbonate filters. The average size of these liposomes was 98.2 ± 22.9 nm ($n = 6$) as determined by quasielastic light scattering analysis. Addition of 20 mol percent anionic or cationic lipids did not alter the average size significantly. The liposome suspensions were 100 mM total lipid in isotonic VBS (10 mM sodium barbital, 145 mM NaCl, pH 7.4).

Serum, complement, and antiserum. Guinea pig serum (Calbiochem Behring, La Jolla, CA) was purchased in a lyophilized form, reconstituted in the buffer provided and stored at −70°C. Human serum was prepared from venous blood from 20 healthy individuals (10 males, 10 females) and stored at −70°C. C1q-deficient serum was purchased from Sigma Chemical Co., St. Louis, MO. Purified guinea pig C1 and C2, and human C1q, C3, and C9 were purchased from Diamedix, Miami, FL. Trypsin-treated C3 was prepared by incubating 50 μl 1 mg/ml purified C3 with 5 μl 1 mg/ml type XIII L-*p*-tosylamino-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) at 37°C for 5 min followed by the addition of 5 μl 5 mg/ml soybean trypsin inhibitor (Sigma). Rabbit antisera to human C3d or C9 were purchased from Calbiochem. Peroxidase-conjugated goat anti-rabbit IgG F(ab')$_2$ fragments were purchased from The Jackson ImmunoResearch Laboratories, Bar Harbor, ME.

Residual total complement hemolytic assays to measure guinea pig and human complement activation by liposomes. Liposome suspensions were serially diluted in DGVB^{2+}. Fifty μl of 5% guinea pig serum in DGVB^{2+} were incubated with an equal volume of liposome suspension at 37°C for 1 h. After the incubation period, the mixtures were diluted with 150 μl of DGVB^{2+} and kept on ice. For studies involving human complement, 50 μl of 25% human serum in DGVB^{2+} was incubated with an equal volume of liposome suspension at 37°C for 30 min. After the incubation period, the mixtures were diluted with 150 μl of DGVB^{2+} and kept on ice.

Residual total complement hemolytic activity of liposome-treated serum was measured according to established methods (27). The hemolysis of heterologous E sensitized with antibody is complement mediated, involving activation via the entire classical pathway. The level of hemolysis is proportional to the level of complement present in the serum. A reduction in the complement hemolytic activity of the serum after incubation with liposomes implies complement consumption and activation by liposomes had occurred. Briefly, sheep E were sensitized with rabbit antibody to E, washed, and suspended at 1.5×10^8 cells/ml in DGVB^{2+}. In triplicates, 50 μl of EA cells were incubated with 50 μl of liposome-treated serum for 30 min at 37°C. Samples were then diluted by the addition of 2 ml of DGVB containing 40 mM EDTA (EDTA-GVB). Unlysed EA cells were pelleted by centrifugation and the amount of hemoglobin released into the supernatant was quantitated spectrophotometrically at 414 nm.

Measurement of guinea pig alternative complement pathway activation by liposomes. Liposome suspensions were serially diluted in DGVB containing 0.5 mM $MgCl_2$ (Mg-DGVB). Ca^{2+}-depleted guinea pig serum was obtained by treating the guinea pig serum with 10% 0.2 M EDTA, pH 7.4, at 37°C for 5 min, followed by supplementing the serum with 10% 0.25 M $MgCl_2$. Fifty μl of 5% Ca^{2+}-depleted guinea pig serum were incubated with an equal volume of liposome suspension at 37°C for 1 h. After the incubation period, the mixtures were diluted with 150 μl of Mg-DGVB and kept on ice.

Residual guinea pig alternative pathway complement hemolytic activity was determined in a Ca^{2+}-free assay using EAC1,4,2 cells as the target cells (27). Immediately before performing the hemolytic assays, the EAC1,4,2 cells were assembled as follows: EA cells (1×10^9 cells/ml in DGVB^{2+}; 1 ml) were incubated with 5000 U of guinea pig C1 for 5 min at 37°C; 5000 U of human C4 were added and the incubation continued for 7 min at 37°C; 250 U of guinea pig C2 were then added and the incubation continued for 7 min at 37°C; finally, the cells were washed and resuspended at 1.5×10^8 cells/ml in Mg-DGVB. In triplicates, 50 μl of EAC1,4,2 cells was incubated with 50 μl of liposome-treated serum for 30 min at 37°C. Samples were then diluted by the addition of 2 ml of EDTA-GVB. Unlysed EAC1,4,2 cells were pelleted by centrifugation and the amount of hemoglobin released into the supernatant was quantitated spectrophotometrically at 414 nm.

Measurement of human alternative complement pathway activation by liposomes. Human alternative complement pathway activation was determined by incubating 500 μl of 20 mM liposomes in EGTA-treated DGVB, containing 0.5 mM $MgCl_2$ (Mg-EGTA-DGVB) with 500 μl of 20% human serum in Mg-EGTA-DGVB at 37°C for 30 min and kept on ice.

Residual alternative pathway human complement hemolytic activity was determined according to published methods (27). By using rabbit E, activators of the alternative pathway of human complement, as target cells in a Ca^{2+}-free hemolytic assay, the activation of the alternative pathway of human complement by liposomes was assessed. Rabbit E were washed and resuspended at 1×10^8 cells/ml in Mg-EGTA-DGVB buffer. In duplicates, 10, 20, 40, 60, 80, and 100 μl aliquots of the serum/liposome incubation mixtures were incubated with 100 μl of rabbit E in a final assay volume of 200 μl for 1 h at 37°C. Mg-EGTA-DGVB was used as the buffer. After the incubation period, 2 ml EDTA-GVB was added to the samples. Unlysed rabbit E were pelleted by centrifugation and the amount of hemoglobin released into the supernatant was quantitated spectrophotometrically at 414 nm.

Liposome lysis fluorescence assay. Liposomes were prepared as above except that the liposomes were in isotonic 20 mM HEPES, pH 7.4, buffer containing 100 mM carboxyfluorescein (purchased from Eastman Kodak and purified according to Weinstein et al. (28)) and the external carboxyfluorescein chromatographically separated using Sephadex G-50 (Sigma) equilibrated with 20 mM HEPES, pH 7.4, 150 mM NaCl buffer. Liposome-treated serum was prepared by incubating 200 μl PC:CHOL:PG or PC:CHOL:CL liposomes in VBS with 1800 μl normal human serum at 0°C for 30 min. One ml aliquots of the liposome/serum incubation mixtures were pipetted into thick walled Beckman 1.5 ml microultracentrifuge tubes (Beckman Instruments, Inc., Fullerton, CA) and centrifuged using the Beckman table microultracentrifuge TL100 for 30 min at 100,000 rpm at 4°C. The top liposome layer was carefully pipetted off and the clear serum was pooled and stored on ice. The liposome lysis fluorescence assay

[3] Abbreviations used in this paper: FMLV, freeze-thawed multilamellar vesicle; PC, egg phosphatidylcholine; PG, egg phosphatidylglycerol; PA, egg phosphatidic acid; PI, bovine liver phosphatidylinositol; PS, bovine brain phosphatidylserine; CL, bovine heart cardiolipin; SA, stearylamine; CHOL, cholesterol; VBS, veronal-buffered saline; EA, sheep E stroma; DGVB, dextrose gelatin VBS; DGVB^{2+}, DGVB containing 50% VBS, 2.5% glucose, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% gelatin.

is detailed elsewhere (28, 29). To 2 ml 25% normal human serum or liposome-treated human serum diluted with $DGVB^{2+}$, 5 μl of carboxyfluorescein-containing PC:CHOL:PG or PC:CHOL:CL liposomes was added and incubated for 30 min at 37°C. The fluorescence of the mixture was read using a Perkin-Elmer LS50 fluorimeter (Perkin-Elmer Corp., Norwalk, CT) with the excitation and emission wavelengths set at 492 nm and 520 nm, respectively. The fluorescence of the total releaseable carboxyfluorescein was measured by adding 100 μl 10% Triton X-100 to the incubation mixtures.

Immunoblot analysis of proteins associated with liposomes after serum incubations. To 2 ml of 100 mM PC:CHOL (55:45) or PC:CHOL:PG (35:45:20) FMLV, 8 ml of undiluted human serum were added and the mixture incubated at 37°C for 30 min. The liposomes were isolated by centrifugation (10,000 × *g*, 10 min, 4°C) and washed five times with 50 ml VBS. The final liposome pellet was resuspended in 2 ml VBS. Proteins from the washed liposomes were extracted according to published methods (30). Briefly, to 1 ml of resuspended liposomes, 4 ml methanol, 1 ml chloroform, and 1.5 ml distilled water were added with vortexing after each addition. The two-phase system generated was separated by centrifugation (20 min, 3000 rpm, Silencer H-103N Benchtop centrifuge (Western Scientific, Vancouver, Canada)). The upper phase was aspirated such that the protein at the interface was left with a slight amount of upper phase. Then 1.5 ml of methanol were added and the protein was precipitated by centrifugation (30 min, 3500 rpm). The supernatant was aspirated and the pellet dried under nitrogen. The dried pellet was resuspended to a concentration of 1 mg/ml in VBS containing 0.5% SDS. Protein concentration was estimated using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Protein separation was performed by SDS-PAGE using the automated electrophoresis apparatus, the PhastSystem (Pharmacia Fine Chemicals, Piscataway, NJ), on precast 7.5% homogenous and 10 to 15% gradient resolving PhastGels (Pharmacia). Prestained SDS-PAGE standards (Diversified Biotech, Newton, MA) were used to estimate the m.w. of the proteins. Electrophoretic transfer of the separated proteins onto nitrocellulose (Nitroplus 2000, Micron Separations, Westboro, MA) was performed using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA) according to the instructions of the manufacturers at constant voltage of 50 V for 45 min. Immunoblot analysis was performed by blocking the nitrocellulose blot with a buffer containing 10 mM Tris, 150 mM NaCl, 3% BSA (Sigma) and 1% normal goat serum (The Jackson ImmunoResearch Laboratories), pH 7.4 (blocking buffer) overnight at 4°C; incubating 0.1% primary antibody in blocking buffer for 2 h at room temperature; incubating 0.02% peroxidase-labeled secondary antibody in blocking buffer for 1 h at room temperature; and finally, developing the labeled bands using 0.3% 4-chloro-1-naphthol (Sigma) in 10 mM Tris, pH 7.4, 150 mM NaCl buffer with 0.018% hydrogen peroxide.

RESULTS

Activation of guinea pig and human complement by phosphatidylglycerol-containing liposomes. Complement hemolytic assays were used to detect activation of the complement system by liposomes. Guinea pig serum was incubated with liposomes composed of PC:CHOL (55:45) and PC:CHOL:PG (35:45:20) for 1 h at 37°C and the residual complement hemolytic activity was quantitated. A reduction in the residual complement hemolytic activity of the serum signifies activation of the complement system by liposomes. The detection of this reduction is sensitive to the initial levels of complement present in the serum. Preliminary studies, therefore, were performed to determine the optimal serum concentration for the hemolytic assay to detect complement consumption (Fig. 1). Diluting the serum with $DGVB^{2+}$ reduced the levels of complement to an optimal range such that when the complement system was not activated, the concentration of the complement proteins was nonlimiting and 100% hemolysis of the target cells occurred; when the complement system was activated, the concentration of the complement proteins was limiting and a reduction in the hemolytic levels occurred. A 1/20 dilution of guinea pig serum was chosen for subsequent hemolysis because this resulted in an optimal concentration for the detection

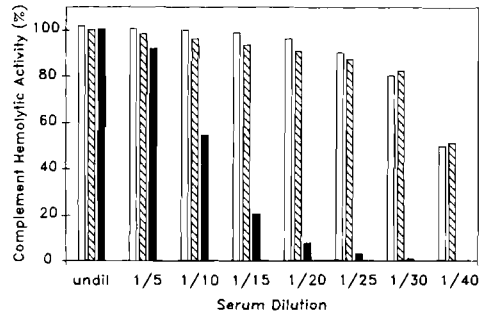

Figure 1. Effect of serum dilution on the sensitivity of the total complement hemolytic assays to detect consumption of guinea pig complement. Fifty μl of undiluted (*undil*) or diluted guinea pig serum in $DGVB^{2+}$ was incubated with an equal volume of $DGVB^{2+}$ (□), 20 mM PC:CHOL (55:45) (▨), or 20 mM PC:CHOL:PG (35:45:20) (■) for 1 h at 37°C. After this incubation period, the mixtures were further diluted with 150 μl $DGVB^{2+}$. The residual complement hemolytic activity of the liposome-treated serum was determined by the complement hemolytic assays and expressed as a percentage of the total hemolytic level determined by lysis of the EA cells with 2 ml distilled water (for experimental details see *Methods and Materials*).

of changes in functional complement levels involving PC:CHOL:PG (35:45:20) liposomes.

Figure 1 shows that the complement hemolytic activity of guinea pig serum exposed to 1 μmol PC:CHOL (55:45) liposomes was the same as serum incubated with $DGVB^{2+}$ buffer. Moreover, Figure 1 shows that PC:CHOL:PG liposomes reduce the ability of guinea pig serum to lyse the EA target cells when compared to serum incubations with $DGVB^{2+}$ buffer. The detection of this consumption was affected by the ratio of the complement concentration in the serum to the amount of liposomes in the incubation mixture (Figs. 1 and 2).

Human complement activation by PG-containing liposomes was also characterized. The hemolytic assays for human serum were optimized essentially as for guinea pig serum and it was found that the optimal dilution of human serum was 1/4. Figure 2 shows that increasing the PG content of the liposomes made the liposomes more potent complement activators. By increasing the surface density of PG, the amount of liposomes required to detect a consumption in the total complement hemolytic activity of human serum was reduced. PG was evidently an important component of complement-activating liposomes as liposomes without PG failed to activate human complement (Fig. 2). Figure 2 also shows that liposomes composed of phospholipids containing unsaturated fatty acyl chains are better activators of human complement than those containing saturated fatty acyl chains. Less surface charge is required in the unsaturated systems to reduce the functional complement levels.

The effect of cholesterol on the complement-activating potential of the liposomes is shown in Figure 3. The inclusion of 45 mol percent cholesterol in both the saturated and unsaturated liposomal systems provided a more potent complement-activating surface.

Complement activation by other charged liposomes. After the demonstration of the functional activation of the complement system by PG-containing liposomes, we wanted to determine whether other negatively charged phospholipids were capable of transforming liposomes into complement-activating membranes. These include CL, PI, PS, and PA, all of which are acidic phospholipids bearing net negative charges at pH 7.4. As shown in Figure 4, the liposomes containing these anionic phos-

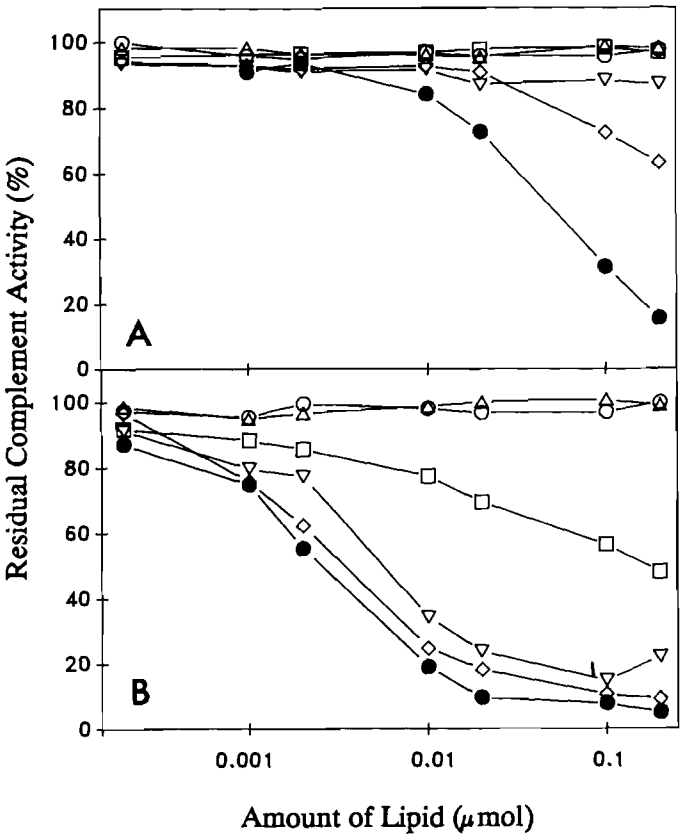

Figure 2. Effect of PG density on human complement activation by saturated and unsaturated liposomes. Liposomes composed of PC (○), PC:PG (9:1) (△), PC:PG (8:2) (□), PC:PG (7:3) (▽), PC:PG (6:4) (◇), or PC:PG (5:5) (●) at various lipid concentrations were incubated with an equal volume of 25% human serum in $DGVB^{2+}$. Their effect on the residual complement hemolytic activity was determined by the complement hemolytic assays and expressed as a percentage of the hemolytic level of human serum incubated with $DGVB^{2+}$. The PC and PG species are dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol for the saturated liposomes (*A*), and dioleioylphosphatidylcholine and dioleioylphosphatidylglycerol for the unsaturated liposomes (*B*).

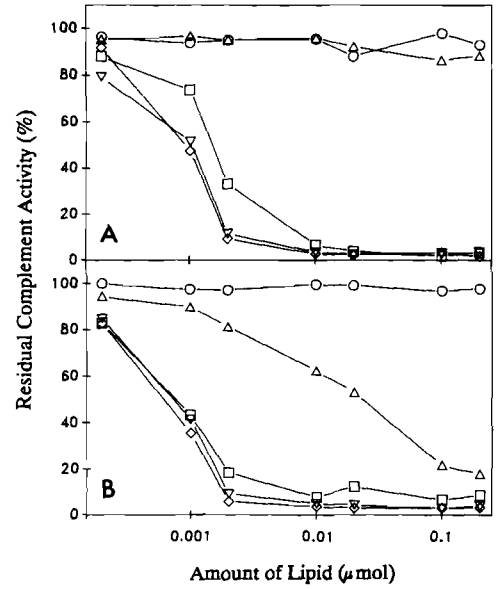

Figure 3. Effect of CHOL on human complement activation by saturated and unsaturated liposomes. Liposomes composed of PC:CHOL (55:45) (○), PC:CHOL:PG (45:45:10) (△), PC:CHOL:PG (35:45:20) (□), PC:CHOL:PG (25:45:30) (▽), or PC:CHOL:PG (15:45:40) (◇) at various liposome concentrations were incubated with 25% human serum in $DGVB^{2+}$. Their effect on the residual complement hemolytic activity was determined by the complement hemolytic assays and expressed as a percentage of the hemolytic level of human serum incubated with $DGVB^{2+}$. The PC and PG species are dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol for the saturated liposomes (*A*), and dioleioylphosphatidylcholine and dioleioylphosphatidylglycerol for the unsaturated liposomes (*B*).

pholipids, present at 20 mol percent of total lipid, were capable of consuming the complement hemolytic activity of guinea pig serum in a dose-dependent manner. Liposomes containing CL were more effective in activating guinea pig complement than the other charged phospholipids. This may arise from the fact that CL bears two negative charges at neutral pH, thus giving rise to a higher surface charge at the same molar ratio.

The importance of surface charge is indicated by the fact that neutral liposomes were not capable of inhibiting the hemolytic activity of guinea pig serum, even at 50 mM lipid concentrations. Liposomes composed of 20 mol percent phosphatidylethanolamine (which is net neutral at pH 7.4) failed to activate guinea pig complement. Moreover, systems with a positive surface charge were capable of consuming guinea pig complement as indicated by the fact that liposomes containing 20 mol percent SA or 20 mol percent 1,2-bis(oleoyloxy)-3-(trimethylammonio)-propane (a nonexchangeable positively charged lipid) activated guinea pig complement.

Figure 5 shows that human complement was activated by similar activator surfaces as guinea pig complement and that surface charge is an important determinant. Notably, much less lipid was required to activate human complement.

Mechanism of complement activation by liposomes. By excluding Ca^{2+} from the assays, alternative pathway activation can be distinguished from classical pathway activation because the initiation complex of the classical pathway, the $C1qr_2s_2$ complex, requires Ca^{2+}. Figure 6 shows that the negatively charged liposomes were not capable of consuming complement in Ca^{2+}-depleted serum. This suggests that the negatively charged liposomes do not activate the alternative pathway and must therefore activate the classical pathway. Under similar conditions, incubation of guinea pig serum with positively charged liposomes resulted in a reduction in the serum C3-C9 levels available to lyse the EAC1,4,2 cells (Fig. 6). This implies that the positively charged liposomes activated the alternative pathway. By using rabbit E, activators of the alternative pathway of human complement, as target cells in the Ca^{2+}-free hemolytic assay, the activation of the alternative pathway of human complement by liposomes was detected. Figure 7 shows that only the positively charged liposomes were capable of reducing the hemolytic activity of human serum under conditions where only the alternative pathway is functional.

To demonstrate that the reduction in residual complement hemolytic activity was not due to the liposomes binding Ca^{2+} thereby reducing the Ca^{2+} ion concentration to levels where the C1 complex is dissociated and not active, binding studies with $^{45}Ca^{2+}$ were done. These studies showed that the liposomes did not significantly deplete the Ca^{2+} concentration of the supernatant in the assays (0.15 mM) (data not shown). Raising the Ca^{2+} concentration to 1.5 mM in the serum/liposome incubations and in the hemolytic assays, however, did abolish the effect of liposomes containing 20 mol percent PG (data not shown). At the higher Ca^{2+} concentrations, it is

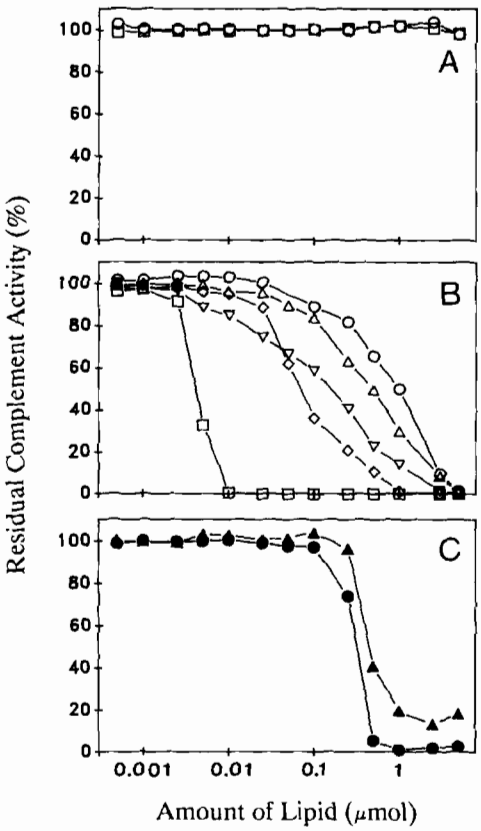

Figure 4. Dose response curves of guinea pig serum incubated with various liposome compositions. *A*, Net neutral liposomes, PC:CHOL (55:45) (○) and PC:CHOL:DPPE (35:45:20) (□); *B*, Negatively charged liposomes, PC:CHOL:PG (35:45:20) (▽), PC:CHOL:PA (35:45:20) ( ), PC:CHOL:PI (35:45:20) (△), PC:CHOL:PS (35:45:20) (○), and PC:CHOL:CL (35:45:20) (□); and *C*, positively charged liposomes, PC:CHOL:SA (35:45:20) (▲) and PC:CHOL:DOTAP (35:45:20) (●) were incubated with 5% guinea pig serum in $DGVB^{2+}$. Their effect on the residual complement activity was determined by complement hemolytic assays and expressed as a percentage of the hemolytic level of guinea pig serum incubated with $DGVB^{2+}$.

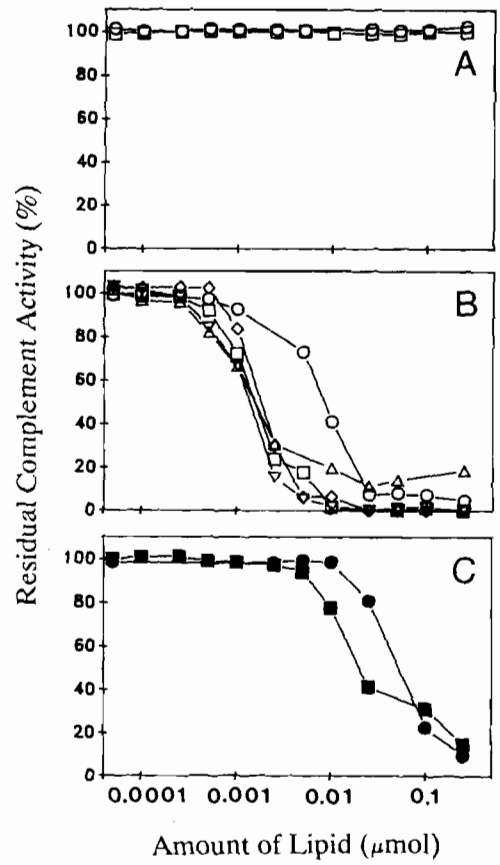

Figure 5. Dose response curves of human serum incubated with various liposome compositions. *A*, Net neutral liposomes, PC:CHOL (55:45) (□) and PC:CHOL:dipalmitoylphosphatidylethanolamine (35:45:20) (○); *B*, Negatively charged liposomes, PC:CHOL:PG (35:45:20) (△), PC:CHOL:PA (35:45:20) (□), PC:CHOL:PI (35:45:20) ( ), PC:CHOL:PS (35:45:20) (○), and PC:CHOL:CL (35:45:20) (▽); *and C, positively charged liposomes*, PC:CHOL:SA (35:45:20) (■) and PC:CHOL:DOTAP (35:45:20) (●) were incubated with 25% human serum in $DGVB^{2+}$. Their effect on the residual complement activity was determined by complement hemolytic assays and expressed as a percentage of the hemolytic level of guinea pig serum incubated with $DGVB^{2+}$.

likely that surface-associated Ca^{2+} has reduced the surface charge of the liposomes, thus inhibiting the interaction of complement proteins and the liposomes.

Membrane lysis resulting from activation of the entire complement cascade was demonstrated using liposomes containing entrapped carboxyfluorescein (Fig. 8). This assay was used to study the effect of preadsorbing the serum with anionic liposomes on the ability of the treated serum to lyse the liposomes. Figure 8 shows that incubating 10 μmol of lipid with 1.0 ml 90% normal human serum for 30 min at 0°C, followed by separation of the liposomes by ultracentrifugation, had very little effect on the ability of the treated serum to subsequently lyse liposomes. To ascertain whether this lysis was complement-mediated, carboxyfluorescein-loaded liposomes were incubated with C1q-deficient human serum. These incubations did not cause an increase in fluorescence over an incubation period of 30 min.

To demonstrate that the consumption of the complement components occurs as a result of complement activation with the subsequent fixation of the converted components onto the liposomal surfaces, the proteins associated with the liposomes after serum incubation were isolated and analyzed by immunoblot analysis. FMLV were used in this study because FMLV were readily isolated from serum by centrifugation. FMLV activated

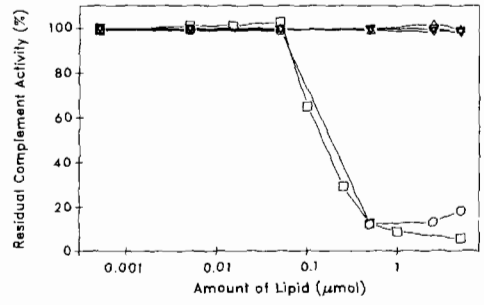

Figure 6. Complement activation by liposomes in Ca^{2+}-depleted guinea pig serum. Liposomes composed of PC:CHOL (55:45) (△), PC:CHOL:PG (35:45:20) (▽), PC:CHOL:PI (35:45:20) ( ), PC:CHOL:SA (35:45:20) (○), and PC:CHOL:DOTAP (35:45:20) (□) were incubated with EDTA-treated, Mg^{2+}-supplemented guinea pig serum. Their effect on the residual complement activity was determined by a Ca^{2+}-free hemolytic assay and expressed as a percentage of the hemolytic level of Ca^{2+}-depleted guinea pig serum incubated with Mg-DGVB.

complement in a similar dose-dependent manner as 100 nm LUV (data not shown). Figure 9 shows that C3b, the activated form of C3, is the predominant molecular species of C3 associated with the complement-activating liposomes. C9 was also detected on the liposomal membranes suggesting that the entire complement pathway was activated and that possibly membrane attack complexes were assembled on the surfaces of the liposomes.

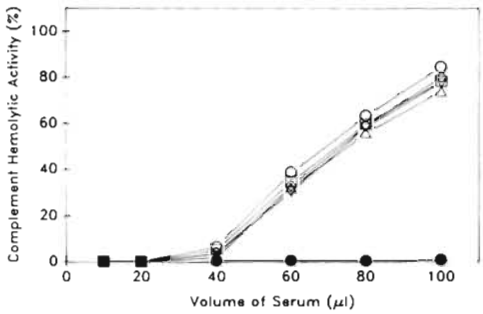

Figure 7. Alternative pathway complement hemolytic activity of human serum incubated with liposomes. Liposomes composed of PC:CHOL (55:45) (▽), PC:CHOL:dipalmitoylphosphatidylethanolamine (35:45:20) ( ), PC:CHOL:PG (35:45:20) (△), PC:CHOL:PA (35:45:20) (□), and PC:CHOL:DOTAP (35:45:20) (●) at 20 mM concentrations were incubated with an equal volume of 20% Ca^{2+}-depleted human serum. A control mixture of Ca^{2+}-depleted human serum and Mg-EGTA-DGVB (○) was included. Various amounts of the liposome/serum mixtures were incubated with rabbit erythrocytes as detailed in *Methods and Materials* to measure the effect of the liposomes on the residual alternative pathway complement hemolytic activity of the serum.

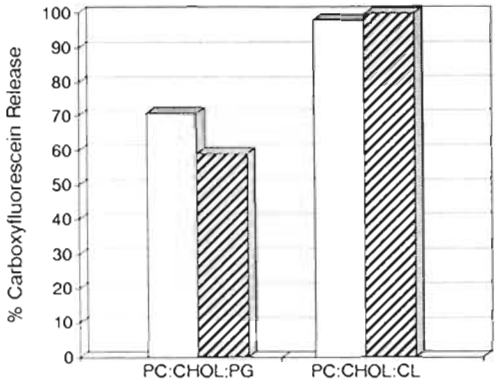

Figure 8. Complement-mediated lysis of liposomes containing entrapped carboxyfluorescein using liposome-treated serum. Two ml of 25% normal human serum (□) or 25% human serum treated with either PC:CHOL:PG or PC:CHOL:CL liposomes (35:45:20) (▨) was incubated with 5 μl PC:CHOL:PG or PC:CHOL:CL liposomes containing entrapped carboxyfluorescein for 30 min at 37°C. The fluorescence due to the released carboxyfluorescein was measured as described in *Methods and Materials* and expressed as a percentage of the total releaseable carboxyfluorescein determined by the addition of 100 μl 10% Triton X-100.

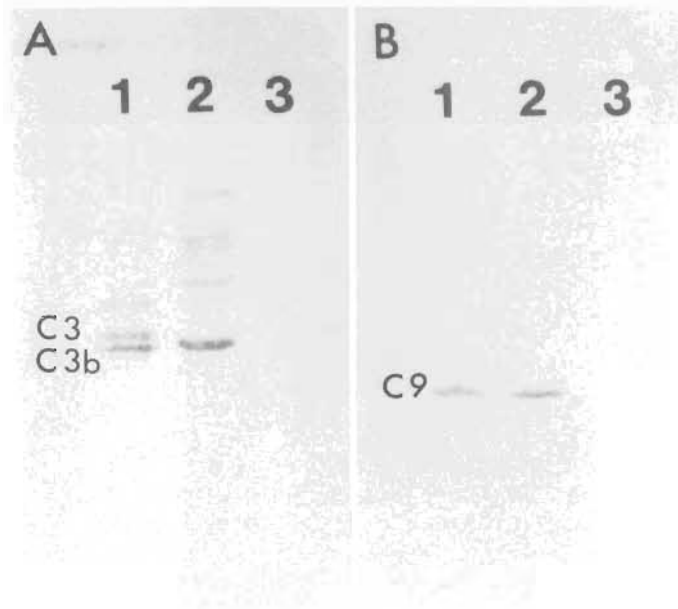

Figure 9. Immunoblot analysis of proteins associated with PG-containing liposomes after exposure to human serum. The proteins associated with PC:CHOL:PG (35:45:20) FMLV (*lane 2*) or with PC:CHOL (55:45) FMLV (*lane 3*) were extracted, electrophoresed on 7.5% homogenous (*A*) and 10 to 15% gradient (*B*) resolving SDS-polyacrylamide gels, blotted onto nitrocellulose, and probed for the presence of activated complement proteins using monospecific antisera to C3d (*A*) or C9 (*B*). *Lane A-1* is trypsin-treated purified C3 and *lane B-1* is purified C9.

These proteins were not detected in extracts of PC:CHOL liposomes as shown in Figure 9. Immunoblot analysis of the proteins with normal rabbit serum did not detect any bands similar to those detected with the specific antibodies.

DISCUSSION

The studies presented here clearly indicate that membranes bearing a net surface charge activate the complement system. Liposomes containing either anionic or cationic lipids reduced the levels of complement present in guinea pig or human serum. Net neutral systems did not affect the functional complement levels.

The nature of the surface charge, whether negative or positive, is important in determining which complement pathway is activated by the liposomes. Under conditions where the classical pathway of complement activation was effectively blocked, positively charged systems were still able to reduce the C3-C9 complement levels in a dose-dependent manner, indicating that positively charged liposomes activated the alternative pathway of complement activation. This is consistent with previous findings that SA-containing liposomes activate complement via the alternative pathway (23) and indicates that guinea pig complement alternative pathway is functional at a 1/20 dilution of guinea pig serum. Negatively charged systems, however, did not consume complement in the absence of Ca^{2+}, suggesting that negatively charged liposomes activated the complement system via the classical pathway. Further evidence to support that positively charged liposomes activated the alternative pathway whereas negatively charged liposomes activated the classical pathway came from measuring residual alternative pathway human complement hemolytic activity. By using rabbit E, activators of the alternative pathway of human complement, as target cells for the hemolytic assays, we showed that only positively charged liposomes were capable of reducing the complement levels of human serum.

The activation of the classical pathway by negatively charged liposomes apparently occurs in an antibody-independent manner because preadsorption of the serum with anionic liposomes had little effect on subsequent complement-mediated liposome lysis. It is not expected that there be a high titer of antibodies directed against the liposomes because these experiments were done in vitro, using isolated guinea pig and human sera. Antibodies to phospholipids, however, have been suggested to be present in normal human serum (31). The generality of the activation by negatively charged phospholipids would suggest that the epitope of the antibody would be the negatively charged phosphate group common to all these anionic phospholipids. PC and phosphatidylethanolamine, however, also possess this phosphate group and thus, we would expect that PC and phosphatidylethanolamine would be recognized by the antibody and would result in antibody-mediated complement activation. We did not observe this. The net negative charge, therefore, directly mediates the liposome-complement interaction in classical pathway activation by negatively charged

liposomes.

With regard to the reduction of functional complement levels after incubating the serum with liposomes, a possibility existed that the surface charge facilitated the "mopping up" of plasma proteins due to electrostatic interactions, thereby decreasing the complement levels in the serum available to lyse the EA target cells. This mechanism is inconsistent with our findings. First, being able to distinguish the two pathways of complement activation implies specificity in the interactions between liposomes and the complement system. Second, the interactions between complement and liposomes could be modulated by altering the physical properties of the membrane (for example, increasing membrane hydrocarbon order; Figs. 2 and 3). Thirdly, immunoblot analysis of the proteins associated with the liposomes after exposure to serum directly demonstrated that degradative products of the complement proteins were associated with anionic liposomal membranes suggesting that the liposomes did represent biologically active surfaces that supported complement activation. Finally, the preadsorption of serum with anionic liposomes at 0°C had little or no effect on the complement-mediated lysis of liposomes containing carboxyfluorescein. Consumption of complement, therefore, was not the result of a general mopping up effect. Alternatively, the negatively charged surfaces could bind Ca^{2+}, limiting its concentration in the incubation mixtures to levels where classical pathway activation and the resultant cytolysis of EA cells cannot occur. This was found not to be the case because PG-containing liposomes did not significantly deplete the supernatant of Ca^{2+}.

Relatively few studies have used liposomes to model the membrane activation of the complement system (16, 17, 22–24, 32). Certain inconsistencies were observed in these studies regarding the influence of lipid composition on complement activation which make it difficult to establish the fundamental properties of complement-activating liposomes. For example, Kovacsovics et al. (16) demonstrated the activation of C1 by liposomes containing CL by [^{125}I]-C1q binding studies; however, liposomes composed of PI in the same study failed to activate C1. Binding of C5b-6 to liposome membranes was shown to require PG or PA; but binding of C5b-6 to PC, PS, or PI was undetectable (33). Liposomes composed of SA have previously been shown to consume complement by the alternative pathway (23); however, these SA-containing systems required the presence of certain glycolipids for C3 conversion. In our study, we have shown that titrating any of the anionic or cationic lipids functionally transformed the liposomes into activators of the complement system.

The activation of the complement system by liposomes was affected by the surface charge density, as well as the dose of liposomes incubated with the serum. This may account for some of the inconsistencies observed in the previous reports because their incubations mostly involved only one concentration of liposomes. Their use of iodinated proteins may also have affected the binding properties and more importantly, the functional activity of the proteins.

Our observations that egg PC:CHOL liposomes containing 20 mol percent DPPE do not activate human or guinea pig complement contrasts with the finding of Mold (24) indicating C3 binding to DPPC:CHOL liposomes containing 20 mol percent DPPE. It is possible that these differences arise from increased membrane order (decreased fluidity) in the saturated DPPC containing systems. This is currently under investigation.

We have demonstrated that when liposomes are incubated with serum, complement proteins become associated with the liposomes. Activation of the complement system by the PG liposomes resulted in the deposition of activated complement products such as C3b on the liposome membranes. These products are known to have significant physiologic roles in the clearance of foreign pathogens. C3b and its degradative products, iC3b and C3dg, have opsonic roles in the clearance of immune complexes. The assembly of the C5b-9 membrane attack complexes results in cell lysis. Inasmuch as these complement components are associated with liposome surfaces, they may play a significant role in the clearance of liposomes from the circulation.

The complement protein-liposome interactions are modulated primarily by surface charge. The physical properties of the membrane including membrane order affect the interactions. The finding that membranes bearing surface charge activate the complement system emphasizes the importance of inherent complement regulatory factors such as decay accelerating factor and homologous restriction factor in preventing autologous complement attack.

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