Unusual Electrostatic Effects on Binding of C1q to Anionic Liposomes: Role of Anionic Phospholipid Domains and Their Line Tension†

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ABSTRACT: The binding of ^{125}I-C1q to anionic liposomes was studied as a function of protein concentration, pH, ionic strength, and anionic lipid composition. The maximum amount of protein bound per micromole of lipid was very sensitive to electrostatic factors, increasing strongly with decreased pH and ionic strength or increased anionic lipid content. The apparent association constant was independent of these electrostatic factors, however, in marked contrast to studies on basic peptide binding to anionic lipid vesicles. Microscopic observations of large unilamellar liposomes containing fluorescently labeled C1q or phosphatidylglycerol demonstrated, under conditions causing strong electrostatic interactions, that C1q and anionic lipids colocalized into domains whose radii of curvature were higher than that of the surrounding lipid. These domains were observed to bud and pinch off into brightly fluorescent vesicles. We propose a model for all of these observations in which the line tension or edge energy at the boundary of the domain resists its increase in circumference as the domain grows by electrostatic effects on binding, eventually resulting in vesiculation. We propose that under favorable electrostatic conditions, as larger domains form the edge energy balances the increases in the electrostatic contribution to binding, resulting in a net binding energy independent of electrostatic factors.

The activation of the classical pathway of human complement occurs by the binding of C1q, either to immunoglobulin or to nonimmunoglobulin activators (*1*). Complement activation by anionic liposomes occurs through the antibody-independent binding of C1q to the surface of the lipid (*2*, *3*). C1q is a 465 kDa glycoprotein consisting of six 131 residue globular headgroups each connected to a long (∼89 residue) triple helical collagen-like stalk that ends at the N-terminus (*4*). From the N-terminus to a hinge region (∼11 nm) the stalks are all associated; beyond the flexible hinge, association ceases and the individual collagen-like triple helices connect independently to the headgroups. Overall, the molecule is pictured as having a form like a bunch of flowers or a beach umbrella (*4*). C1q is found in human serum at a concentration of 80−100 mg/L (*1*). Most C1q normally circulates as part of the C1 complex with two each of the C1r and C1s subunit proteins in a calcium-dependent association in the stalk region (*5*). In the presence of antibody, C1q binds through the globular domains (*1*). In the absence of antibody, C1q can bind directly through the collagen-like stalks to a variety of substances, including a number of proteins, polyanions, DNA, and many different cell types (*6*−*8*). Antibody-independent C1q binding can provide efficient initiation of an immune response before antibodies are present.

The activation of complement by anionic liposomes is also of interest because liposome efficacy as a pharmacological delivery vehicle can be lost due to complement-mediated liposome opsonization and phagocytosis (*9*−*14*) or to membrane attack complex-induced loss of liposome stability and subsequent leakage of entrapped substances (*15*, *16*). In particular, complement activation is potentially present in systems such as the cardiolipin-based and PG[1]-based formulations of doxorubicin (*17*, *18*), PG-based amphotericin B preparations (*19*), and liposomes that are specifically targeted to the reticuloendothelial system (*20*, *21*).

While little is known about the interaction between anionic liposomes and C1q, it has been proposed that negative surface charge and the presence of cationic sequences are required for the binding of C1q to nonimmunoglobulin targets through the collagen-like stalks (*22*−*25*). The goal of this study was to make fundamental measurements of C1q

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[1] Abbreviations: C1q-DS, C1q-deficient serum; CH, cholesterol; CH50, functional complement hemolytic 50; CL, cardiolipin derived from bovine heart; CL20, liposomes composed of PC/CH/CL at 35:45:20 mol %; CL40, liposomes composed of PC/CH/CL at 15:45:40 mol %; ELISA, enzyme-linked immunosorbent assay; IEP, isoelectric point; LUV, large unilamellar vesicle; MARCKS, myristoylated alanine-rich C kinase substrate; MLV, multilamellar vesicle; NHS, normal human serum; PC, phosphatidylcholine derived from egg; PKC, protein kinase C; PG, phosphatidylglycerol derived from egg; PG40, liposomes composed of PC/CH/PG at 15:45:40 mol %; PS, phosphatidylserine; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

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binding to negatively charged liposomes as a function of pH, ionic strength, and anionic lipid composition in order to determine the extent of electrostatic involvement in the binding interaction. We also made observations of the accumulation into local membrane domains of negative lipid and C1q utilizing fluorescently labeled material and the subsequent loss of these domains into the suspending medium via vesiculation. Unexpectedly, we found that the apparent association constants interpreted from the binding isotherms were virtually independent of electrostatic factors but that the maximum amount bound was clearly determined by electrostatic interactions. We have interpreted this unusual behavior in terms of a model based on protein-induced domain formation and the effect of the domain boundary line tension on reducing the free energy of C1q association as the domains form.

MATERIALS AND METHODS

Purification of C1q from Human Plasma. C1q was purified from acid citrate dextrose-anticoagulated human plasma obtained from the Canadian Red Cross using the method of Tenner et al. (*26*) with the following modifications. First, prior to chromatography on BioRex 70 (BioRad, Hercules, CA), serum lipids were reduced by centrifugation at 12500*g* for 30 min at 4 °C. The serum was then removed by pipet from underneath the floating lipid layer. Second, the BioRex column was run at pH 7.1 rather than pH 7.3 in order to increase retention of C1q on the column. Isolated C1q was assessed for purity by SDS/PAGE with silver staining and Western blotting. C1q function was tested using a C1q ELISA which assessed the ability of purified C1q to bind aggregated whole human IgG. The C1q ELISA functional test was the same as the binding ELISA we have previously reported (*2*), with the exception that no liposomes were present. To assess the hemolytic capacity of purified C1q, we altered the standard hemolytic assay (*27*) such that the test C1q was added back at 50 μg/mL to C1q-depleted serum which was generated during the isolation of C1q from human plasma. After serially diluting the C1q-DS/C1q mixture, antibody-sensitized sheep red blood cells were added. The amount of lysis was determined according to Mayer (*27*), and CH50 values were derived by regression. C1q-DS alone had no hemolytic activity. When purified C1q was added back at a nearly native concentration, the ELISA result and hemolytic activity achieved were comparable to that of normal human serum. Purified C1q was stored in 50 mM Tris with 0.5 M NaCl at −80 °C in aliquots and was used without repeated freeze−thaw for up to one year from the isolation date.

Radiolabeling of C1q. Purified human C1q, isolated as above, was radiolabeled using the Iodogen method (Pierce Chemical, Rockford, IL). When commercial C1q (Cedarlane, Hornby, Ont.) was used, the glycerol in the storage buffer was removed by dialysis prior to radiolabeling. The molar ratio of $Na^{125}I$ to C1q was either 1:4 or 1:10, depending on the specific activity desired. Radiolabeled C1q was assessed for purity by SDS/PAGE and autoradiography and for function using the C1q hemolytic assay described above. Specific activity of the labeled C1q was determined using the BCA assay (Pierce) on the iodinated material, calibrated with commercial C1q to measure protein concentration. ^{125}I-C1q was stored at 4 °C and was used for up to 2 months from the labeling date.

C1q-Derived Peptides. A reported binding site for non-immunoglobulin activators of C1q lies between residues 14 and 26 of the C1q A-chain (*25*). For these studies, this peptide, $C1qA_{(14-26)}$, was synthesized by the Biotechnology Protein Service Laboratory of the University of British Columbia; the peptide sequence is A-G-R-P-G-R-R-G-R-P-G-L-K.

Preparation of Liposomes. Liposomes were made as previously described (*28*). Dried lipids were resuspended in buffer (1.8 mM sodium barbital, 3.1 mM barbituric acid, 11% sucrose, with 20, 100, or 145 mM NaCl depending on the ionic strength desired). Multilamellar vesicles (MLVs) were extruded under pressure through two stacked 400 nm diameter pore size polycarbonate filters (Costar, Cambridge, MA) (extruder from Lipex Biomembranes, Vancouver, Canada), producing liposomes with a mean diameter of 240 ± 38 nm (by Nicomp Submicron Particle Sizer, Model 270, Particle Sizing Systems, Inc. Santa Barbara, CA). Unextruded MLVs were used for particle electrophoresis. Liposome phospholipid content was determined using a colorimetric phosphate assay (*29*). Very large unilamellar liposomes for fluorescence microscopy were made according to Needham and Evans (*30*) and were composed of PC/CH/PG/NBD/PG at 15:45:37:3 mol %. Cholesterol was from Sigma (St. Louis, MO), and all other lipids, including NBD/PG, were from Avanti Polar Lipids, Inc. (Alabaster, AL).

Equilibrium C1q Binding Assay. Equilibrium binding measurements were made with liposomes suspended in C1q and buffer. Reaction mixtures consisted of 15 μL of C1q/^{125}I-C1q mixture at the appropriate NaCl concentration, 5 μL of sucrose-containing ∼240 nm liposomes, and 80 μL of diluting buffer containing 1.8 mM sodium barbital, 3.1 mM barbituric acid, 7.2% D-glucose, and 77.9 mM, 25 mM, or no NaCl to achieve a final ionic strength of 145, 100, or 20 mM NaCl, respectively. The pH was adjusted as required by the addition of 0.2 M HCl.

Following a (typical) 20 min reaction at room temperature (RT; incubation times of 1−60 min were tested), 40 μL of reaction mixture was layered onto 180 μL of an intermediate density separating buffer (1.8 mM sodium barbital, 3.1 mM barbituric acid, 4.5% sucrose, 2.9% D-glucose, and NaCl at 145, 100, or 20 mM) in 5 × 20 mm polyallomer tubes. After centrifugation for 30 min at 166300*g*, >90% of the liposomes were pelleted. Tubes were then frozen and sliced just above the pellet into two pieces. The pellet slice contained liposome-bound C1q, while the supernatant slice provided a measure of the equilibrium C1q concentration. Tube slices were counted in an LKB Wallac gamma counter (Compugamma Model 1282). As a control, tubes containing only the C1q mixture were run in parallel (C1q control). The amount of C1q spun down in this control tube (≤8% of total) was subtracted from the binding values for the liposome/C1q reaction tubes. The amount of C1q bound to liposomes was calculated as follows: μg of C1q bound = (cpm_{pellet} − $cpm_{C1q\ control}$)/specific activity of C1q mixture.

Binding Data Treatment. All binding isotherm data were fit to the following fundamental binding equation:

$$Y = (P_1P_2X)/(1 + P_2X) \quad (1)$$

where Y = amount of C1q bound per micromole of lipid, P_1 = maximum amount of C1q bound at saturation, P_2 =

apparent association constant or affinity constant, K_a (M^{-1}), and X = equilibrium concentration of C1q (M).

Data fitting was by nonlinear least squares (*31*), utilizing the ORIGIN package (Microcal Software Inc., Northampton, MA).

Assessment of Reversibility of C1q/Liposome Binding by Particle Electrophoresis. Particle electrophoresis was used to measure the mobility of CL20 MLVs alone, in the presence of C1q, and after washing. A thermostated cylindrical chamber apparatus equipped with water immersion optics was used; liposome mobilities were measured as previously described (*32*). CL20 MLV mobilities were first measured in the absence of C1q and in 1.8 mM sodium barbital, 3.1 mM barbituric acid, and 100 mM NaCl buffer at pH 7.0. The CL20 MLVs (0.36 mM total lipid) were then incubated for 40 min at RT with 0.134 μM C1q in a total volume of 780 μL. Buffer was added to bring the reaction mixture volume to 2.8 mL in order to flood the electrophoresis chamber, and vesicle mobilities were measured. The diluted reaction mixture was removed from the electrophoresis chamber. MLVs were washed by the addition of buffer (2 mL) and centrifugation at 1800*g* for 6 min. The liposome pellet was reconstituted in 2.8 mL of fresh buffer, and liposome mobilities were measured as above. A second wash was performed in the same manner, and the mobility of the washed CL20 vesicles was measured.

Assessment of C1q Integrity at Low pH by Particle Electrophoresis. Particle electrophoresis was used to demonstrate the reversibility of the binding of C1q to CL20 MLVs in 145 mM NaCl under cycled pH conditions. CL20 MLV mobilities were first measured in the absence of C1q in 1.8 mM sodium barbital, 3.1 mM barbituric acid, and 145 mM NaCl buffer, at pH 5 and 7.0. Mobilities were then measured for CL20 MLVs (0.36 mM total lipid) following incubation for 40 min at RT with 0.134 μM C1q at pH 5 in a total volume of 780 μL. To test reversibility, we removed the mixture from the electrophoresis chamber, adjusted the pH to 7 by the addition of 0.5 M NaOH, and again measured vesicle mobilities. The mixture was then removed from the electrophoresis chamber, pH was adjusted back to pH 5 by the addition of 0.2 M HCl, and mobility was measured a third time.

Domain Formation Assessment by Phase Contrast and Fluorescence Microscopy. Giant liposomes in the size range of 4−8 μm were viewed by phase contrast or fluorescence microscopy on a Zeiss AXIOVERT (×63 oil immersion objective and ×2 condenser magnification) or a NIKON Labophot 2A (×100 oil immersion objective). On the Zeiss microscope images were taken with a 35 mm camera. The NIKON microscope is fit with a Pixera Professional digital camera (Los Gatos, CA) for real time imaging. Giant LUVs tend to stick and spread on the glass slides, especially after the addition of positively charged protein or poly-DL-lysine hydrobromide (Sigma). As a preventive procedure the glass was coated with dimethyldichlorosilane (Sigma).

A fraction of 8% of the total PG being NBD-labeled on the acyl chain proved to be a concentration where no detectable self-quenching occurred and the LUVs were easily visible under the microscope. To monitor C1q accumulation, we labeled it with a fluorescein analogue dye, Alexa 488. Five hundred microliters of a 1 mg/mL solution of purified C1q (Calbiochem) was labeled according to the protocol supplied by the manufacturer (Molecular Probes, Eugene, OR). The reagent mixture was stirred in the cold overnight and separated from unbound dye on the column provided the next day at RT.

To observe domain formation of PG on giant liposomes in the presence of poly-DL-lysine (Sigma: 2400 Da) or C1q, we used essentially the methods described by Haverstick and Glaser (*33*). No agarose was included in any experiments. Domain formation, defined as accumulation of negatively charged lipid in the presence of positively charged peptide or protein, was characterized as an increase in lipid-bound NBD fluorescence and/or an increase in C1q-bound Alexa 488 fluorescence. The final poly-DL-lysine and C1q concentrations in domain formation studies were 100 μM and 36 or 100 nM (as indicated), respectively, at 85 μM total lipid concentration.

When liposomes and C1q or peptide were mixed in a test tube, domain formation was observed by the time the sample was mounted on the microscope, about two minutes. In some cases, liposomes and peptide were mixed on the slide to directly view the interaction. An indication of peptide binding was immediately observed, and pinching off of peptide-covered vesicles was seen after about 9 min.

RESULTS

C1q purified by the method described was fully functionally active in both the native and radiolabeled forms; radiolabeled C1q retained its original hemolytic activity for up to 2 months. Purified C1q binding to anionic liposomes was measured as a function of incubation time, C1q concentration, anionic lipid content, pH, and ionic strength. The binding experiments were considered to provide equilibrium binding data for two reasons. First, the time course of binding to CL20 liposomes at either pH 4 or pH 7.2 indicated that maximal binding occurred within one minute under all assay conditions. Second, the amount of C1q bound per amount of lipid was independent of the conditions used in the centrifugation separation step. Whether the liposomes were spun down at 166300*g* for 30 min or at 99800*g* for 45 min, the amount of C1q bound per amount of lipid was the same.

Reversibility of C1q Binding to Anionic Liposomes. To determine if the interaction between C1q and anionic liposomes was reversible, we monitored C1q binding to CL20 MLVs before and after washing by particle electrophoresis. This experiment was carried out at slightly subphysiologic ionic strength (100 mM NaCl) in order to enhance C1q/liposome interactions. In the absence of C1q, CL20 MLVs had a high negative mobility in the applied electric field ($-3.97 \pm 0.18 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}; $n = 20$ and mean ±1 SD reported for all measurements). When C1q was incubated with liposomes prior to vesicle electrophoresis, the liposomes showed some aggregation and their mobilities decreased drastically due to the reduced negative surface charge contributing to the zeta potential associated with C1q binding ($-0.84 \pm 0.36 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}). After one buffer wash, liposome mobilities returned to the original level for CL20 MLVs alone ($-3.49 \pm 0.58 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}), indicating that liposome-bound C1q had dissociated. These results demonstrate that C1q binding to anionic liposomes is reversible under these experimental conditions.

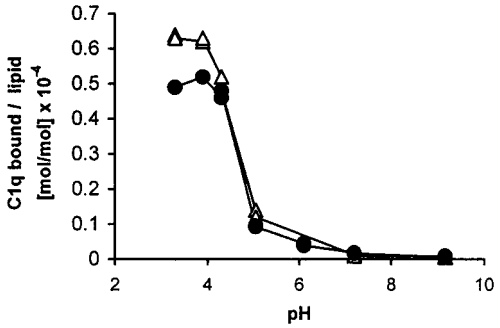

FIGURE 1: pH dependence of C1q binding to anionic liposomes. C1q binding to CL- or PG-containing liposomes was measured as a function of pH using the equilibrium C1q binding assay with purified C1q in barbital buffer containing 145 mM NaCl. These experiments were conducted at subsaturating concentrations of C1q. Binding data for CL20 liposomes are represented by ●, and data for PG40 liposomes are represented by Δ. Typical uncertainties in all binding data in Figures 1−3 are ±5%.

pH Dependence of C1q Binding to Anionic Liposomes. C1q binding to cardiolipin or phosphatidylglycerol-containing liposomes was found to be highly pH-dependent. The two binding curves in Figure 1 are representative of five experiments carried out using different preparations of C1q and in barbital or Tris buffers (other data not shown).

Binding of Purified C1q to Anionic Liposomes at Low pH. To examine whether C1q was altered after exposure to low pH, we monitored C1q/CL20 liposome interactions at physiologic ionic strength (0.145 M NaCl) by particle electrophoresis under cycled pH conditions. For a single reaction mixture of C1q and CL20 MLVs, the effect of C1q binding on lipid mobility was assessed at pH values of 5 and 7 and then at pH 5 again. In the absence of C1q, vesicle mobilities did not change significantly when the pH was adjusted from 5 to 7 (-3.04 ± 0.16 to $-3.32 \pm 0.09 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}). In the presence of C1q, liposome mobilities varied dramatically from pH 5 to 7. At pH 5, the mean mobility, $+0.38 \pm 0.04 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}, changed sign relative to the control, indicating a strong interaction of positively charged C1q with CL20 liposomes. When the pH was raised to 7, C1q no longer affected the mobility ($-3.50 \pm 0.16 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}). When the pH was again lowered to 5, the initial mobility value was observed ($+0.38 \pm 0.10 \times 10^{-4}$ cm^2 volt^{-1} s^{-1}). These results argue that the region of C1q involved in binding anionic liposomes was not altered as a result of low pH exposure in a way which produced irreversible binding. Hence, the interaction responsible for the mobility decrease was reversible with respect to pH dependence.

Since C1q binding to anionic liposomes was maximum at pH 4, a set of binding assays was conducted at pH 4 to obtain isotherms under conditions that promoted strong interactions. C1q binding data for liposomes with 20 or 40 mol % CL and for liposomes with 40 or 55 mol % PG are shown in Figure 2. Even at pH 4, neutral PC/CH liposomes failed to bind detectable amounts of C1q. For anionic liposomes, the level of C1q binding was dependent on the negative lipid composition. When the CL content was raised from 20 to 40 mol %, a 4-fold increase in C1q binding was observed although the apparent association constant was if anything decreased (Table 1). Increasing the PG content from 40 to 55 mol % resulted in almost twice the amount of C1q bound

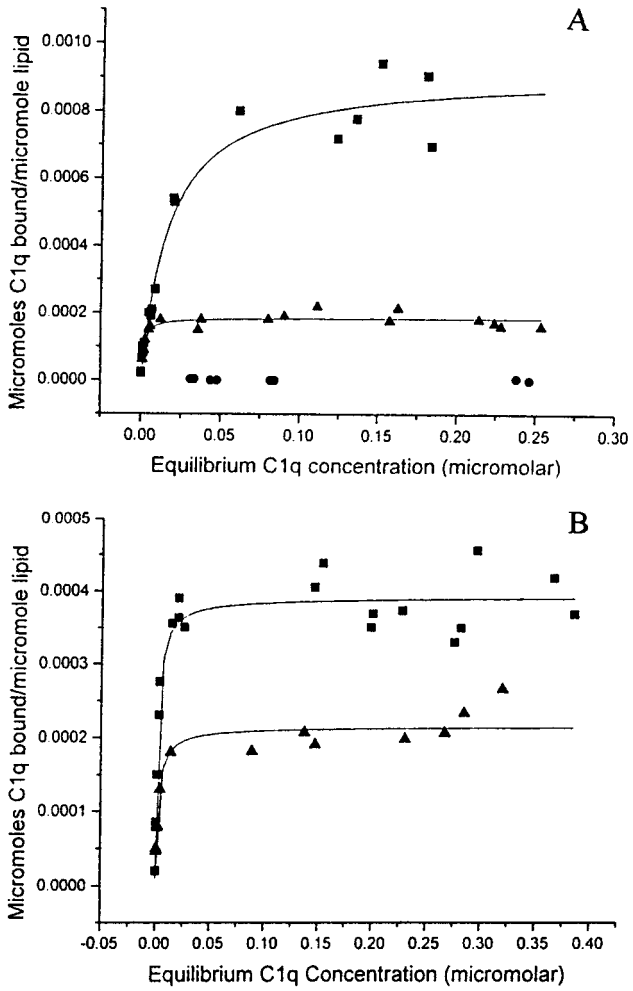

FIGURE 2: C1q binding to anionic liposomes at low pH. Isotherms for C1q binding to 240 nm liposomes composed of PC/CH (●), PC/CH/CL (35:45:20 mol %) (▲), and PC/CH/CL (15:45:40 mol %) (■) at pH 4 and 145 mM NaCl were obtained using the equilibrium C1q binding assay (A). Isotherms for C1q binding to 240 nm liposomes composed of PC/CH/PG (15:45:40 mol %) (▲) and CH/PG (45:55 mol %) (■) at pH 4 and 145 mM NaCl are shown in panel B. Solid lines are plots of eq 1 with the best fit parameters from Table 1.

at saturation, again with virtually no effect on the apparent association constant. A summary of C1q binding parameters at pH 4 derived from nonlinear least-squares fit of the data in Figure 2 is presented in Table 1B.

Effect of Ionic Strength on C1q/Liposome Binding. The dependence of C1q/liposome binding on ionic strength was demonstrated using the equilibrium C1q binding assay on both extruded liposomes (average diameter 240 nm) and on MLVs (Figure 3). Neutral PC/CH extruded and MLV liposomes failed to bind detectable amounts of C1q even at low ionic strength (20 mM NaCl). The maximal amount of C1q binding to anionic liposomes increased strongly as ionic strength was decreased both for extruded CL20 liposomes (panel A) and for CL20 MLVs (panel B). For MLVs, as we have previously shown for extruded liposomes (*23*), small but nonzero levels of C1q binding were measured at 145 mM NaCl. At 100 mM NaCl, extruded liposomes bound 80 times more C1q and MLVs bound an estimated 10 times more C1q at saturation than at 145 mM. At 20 mM NaCl, virtually all of the C1q added bound to CL20 vesicles and

Table 1: Apparent Association Constants and Saturation Values for C1q Binding to Liposomes[a]

	liposome composition	χ^2 for nonlinear fit	$K_a \pm$ ESE[b] (M^{-1})	saturation binding: μmole of $C1q_{bound}/\mu$mole of exposed lipid $\pm$ ESE[b]	saturation binding: molecules of $C1q_{bound}$/vesicle
			(A) pH = 7.2		
	PC/CH/CL (35:45:20)	6.4×10^{-14}	$4.5 \pm 0.8 \times 10^8$	$6.3 \pm 0.3 \times 10^{-6}$	1.6
$I = 145$ mM	PC/CH/CL (25:45:30)	1.3×10^{-13}	$5.1 \pm 1.0 \times 10^8$	$1.9 \pm 0.1 \times 10^{-5}$	4.9
	PC/CH/CL (15:45:40)	1.0×10^{-11}	$4.7 \pm 0.6 \times 10^8$	$1.7 \pm 0.07 \times 10^{-4}$	44
			(B) pH = 4.0		
	PC/CH (55:45)	nd	0	0	0
	PC/CH/CL (35:45:20)	3.1×10^{-10}	$7.5 \pm 1.4 \times 10^8$	$5.3 \pm 0.15 \times 10^{-4}$	137
$I = 145$ mM	PC/CH/CL (15:45:40)	4.2×10^{-9}	$6.1 \pm 1.0 \times 10^7$	$2.6 \pm 0.11 \times 10^{-3}$	672
	PC/CH/PG (15:45:40)	3.9×10^{-10}	$4.2 \pm 0.81 \times 10^8$	$6.0 \pm 0.22 \times 10^{-4}$	155
	CH/PG (45:55)	9.9×10^{-10}	$5.5 \pm 0.96 \times 10^8$	$1.1 \pm 0.03 \times 10^{-3}$	284
			(C) pH = 7.2		
	size of PC/CH/CL (35:45:20)	χ^2 for nonlinear fit	$K_a \pm$ ESE[b] (M^{-1})	μmole of $C1q_{bound}/\mu$mole of exposed lipid $\pm$ ESE[b]	molecules of $C1q_{bound}$/vesicle
$I = 145$ mM	240 nm	6.4×10^{-14}	$4.5 \pm 0.8 \times 10^8$	$6.3 \pm 0.3 \times 10^{-6}$	1.6
$I = 100$ mM	240 nm	4.7×10^{-10}	$1.4 \pm 0.6 \times 10^8$	$4.9 \pm 0.5 \times 10^{-4}$	127
$I = 20$ mM	240 nm	7.5×10^{-9}	$5.9 \pm 1.6 \times 10^8$	$1.3 \pm 0.2 \times 10^{-3}$	336
$I = 145$ mM	mlv	3.0×10^{-10}	$3.8 \pm 1.6 \times 10^7$	$2.2 \pm 0.3 \times 10^{-4}$	nd
$I = 100$ mM	mlv	2.2×10^{-7}	$2.2 \pm 1.6 \times 10^8$	$2.5 \pm 0.3 \times 10^{-3}$	nd
$I = 20$ mM	mlv	8.8×10^{-8}	$2.2 \pm 0.2 \times 10^8$	$1.2 \pm 0.05 \times 10^{-2}$	nd

[a] Saturation binding and apparent association constants, K_a, for CL20, CL30, and CL40 liposomes at pH 7.2 are from Bradley et al. (*23*) and are presented here for comparison (A). Apparent association constants and C1q saturation values for C1q binding to 240 nm liposomes at pH 4 (B) and for C1q binding to 240 nm CL20 liposomes and to CL20 MLVs at pH 7.2 at ionic strengths (I) of 0.145, 0.100, and 0.020 M (panel C) were derived from nonlinear least squares fits to the fundamental binding equation (eq 1). The values of χ^2 given are the sums of the squares of the unweighted differences between the theoretical curve and the experimental points. The number of C1q molecules per liposome was calculated assuming an area per lipid molecule of 0.7 nm^2. The fraction of lipid exposed was calculated as described in the legend to Figure 3. [b] ESE: estimate of standard error. nd: not determined.

saturation was not achieved at the concentrations tested. The nonlinear curve fit predicted saturation at a level about 5 times that measured at 100 mM. No change in apparent binding constant was predicted, however. Saturation binding values and apparent association constants for these data are summarized in Table 1C.

C1q-Induced Anionic Phospholipid Domains. LUVs containing a mixture of NBD-labeled and unlabeled PG (PC/CH/PG/NBD/PG 15:45:37:3), at total negative lipid compositions (40 mol %) that produced strong C1q binding, were observed to undergo a transformation in the distribution of fluorescence when exposed to proteins known to bind to the liposome surface. In the absence of external agents, the LUVs exhibited a uniform fluorescence, bright at the periphery because of the geometry of the curved membrane and the uniform distribution of the NBD/PG (Figure 4A). Brief exposure (less than 5 min) to 100 μM poly-DL-lysine 2400, however, resulted in rapid accumulation of the fluorescing anionic PG in one region of the vesicle (Figure 4C). In phase contrast such vesicles were clearly seen to have changed radius of curvature in the region which exhibited the high fluorescence (Figure 4B).

When the same preparation of liposomes was exposed to a sufficient concentration of C1q to saturate the binding isotherm (Figure 2B), again the fluorescence distribution went from being uniform over the periphery of the optical section observed in the absence of C1q (Figure 5A) to a punctate distribution in its presence (Figure 5B). Addition of 140 μM $C1qA_{(14-26)}$, a basic peptide of net charge +5 that inhibits binding of C1q to anionic liposomes (*23*), also induced highly localized fluorescence (Figure 5C). Similar results were observed up to physiological ionic strength when C1q was added at higher concentrations.

Alexa 488 labeling of C1q allowed the distribution of protein on the liposome surface to be observed, in some experiments simultaneously with the NBD-labeled lipid distribution when both labeled species were present in the samples. Because of relatively rapid photobleaching of NBD under the illumination conditions required to provide clear images, it was possible to distinguish the much more stable Alexa 488 signal from the rapidly bleached NBD signal. Comparison with the phase contrast image in either case showed that the region with the higher radius of curvature than the liposome body contained intense fluorescence. Hence, it was clearly observed that the anionic lipid and C1q were colocalized in every case in which accumulation of either was observed. Figure 6 shows the phase contrast (I) and fluorescence (II) images of anionic (PG40) LUVs exposed to a saturating concentrations of Alexa 488-C1q in barbital-buffered 20 mM NaCl. Again the fluorescence is seen to accumulate in a protrusion extending out from the remainder of the vesicle. Phase contrast images show a progressive change in structure of the liposome, culminating (Figure 6D) in the breakup of the LUV into much smaller vesicles. One such vesicle is clearly seen in Figure 6D as the bright point in the fluorescence image in the lower center-right quadrant and as the dark vesicle near the top of the lowest large vesicle form at the corresponding location in the phase contrast image. Most of the large vesicle does not fluoresce.

Figure 7 shows another pair of images from the same preparation as that from which Figure 6 was derived, captured less than 5 min after the addition of C1q to the vesicle suspension. In this case, most of the fluorescence is concentrated in small, bright vesicles seen throughout the fluorescence field. Few large vesicles are observed and none

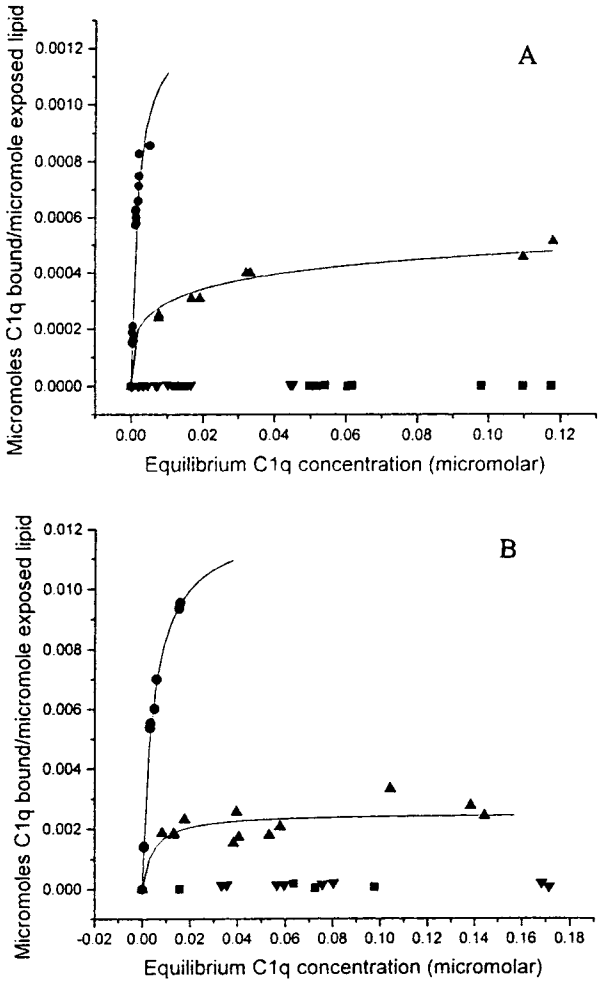

FIGURE 3: C1q/CL20 binding: effect of ionic strength. The equilibrium C1q binding assay was used to measure C1q binding to extruded 240 nm CL20 liposomes (A) and to CL20 MLVs (B) at 20 (●), 100 (▲), and 145 mM (▼) NaCl. PC/CH (55:45 mol %) extruded liposomes and MLVs were also assessed for C1q binding at 20 mM NaCl (■). Because the lamellarity differed between extruded and nonextruded vesicles, the amount of C1q bound to vesicles was expressed as micromoles of C1q bound per micromole of exposed lipid. For the 240 nm diameter extruded liposomes, the amount of exposed lipid was taken to be 35% of the total lipid (based on ^{31}P NMR data in ref *2*). For MLVs, the amount of exposed lipid was taken to be 5% of the total lipid (*62*). Solid lines are plots of eq 1 with the best fit parameters from Table 1.

show bright peripheral fluorescence. Comparison of the phase contrast and fluorescence images shows that none of the large, seemingly intact vesicles show much fluorescence. On these LUVs fluorescence occurs in small spots or chains.

DISCUSSION

C1q Binding to Anionic Liposomes. C1q consists of six identical chains each with a globular headgroup attached to a triple helical collagenous stalk. Its isoelectric point is estimated to be in the range 6.1−7.0 (*34*). Solution studies by hydrodynamic and neutron diffraction methods on human C1q (*35*) provide estimates of its size in solution that allow a calculation of the area it might occupy when associated with a surface. An acceptable average for the maximum diameter is ∼35 nm, which represents the size of the six headgroup region that binds to antibodies to initiate the classical pathway of complement activation. From this we estimate a maximum area per molecule of approximately

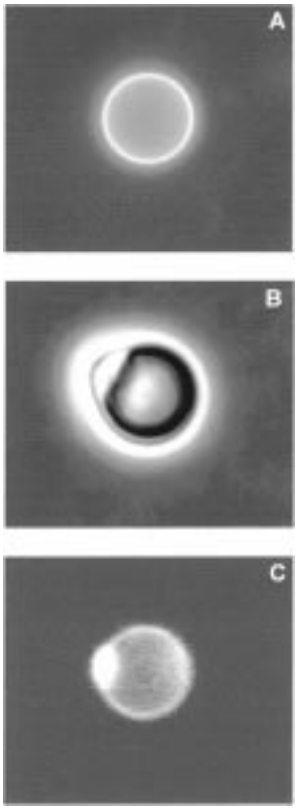

FIGURE 4: Domain formation by poly-DL-lysine. Panel A shows a fluorescence micrograph of an untreated giant liposome that is visualized by acyl chain labeled NBD/PG (8% of total PG). Panels B and C are a phase contrast and a fluorescence micrograph, respectively, showing the interaction of poly-DL-lysine (2500 Da) with a giant liposome ∼2 min after the addition of poly-DL-lysine (100 μM final concentration) in veronal buffer (20 mM NaCl, pH 4) to giant liposomes (85 μM total lipid concentration). In phase contrast microscopy structural changes of the liposomes, that is, changes in curvature of parts of the membrane, were seen (B). Addition of poly-DL-lysine caused accumulation of NBD label in the bulging area, indicating domain formation of the negatively charged lipid. Images A and B were taken on the Zeiss and C on the Nikon microscope. The size range of liposomes shown in the figure is 5−8 μm.

1200 nm^2. The molecule is known to self-associate to some extent in low ionic strength media (*26*).

The C1q/anionic liposome binding reaction was found to be rapid and reversible in 0.1 M NaCl and pH 7 by particle electrophoresis. The reversibility is unusual in protein adsorption studies, as most surfaces that do not contain specific receptors bind protein virtually irreversibly (*36*). The electrophoretic mobility, the velocity of a vesicle in an applied electric field, is proportional to the zeta potential, the average electrostatic potential at the hydrodynamic surface of the particle. When C1q is added, the binding of the protein to liposomes is detected as a decrease in the liposome electrophoretic mobility due to replacement of some of the liposome surface charge by charges on bound C1q. Consequently, the decrease in electrophoretic mobility is directly related to the amount of C1q bound. Quantitative interpretation of the changes in terms of the surface concentration of C1q is not possible, however, since little is known about the detailed orientation of the bound species. The interaction of C1q with CL20 liposomes was found to be reversible since after one washing step the mobility change associated with C1q binding was no longer observed.

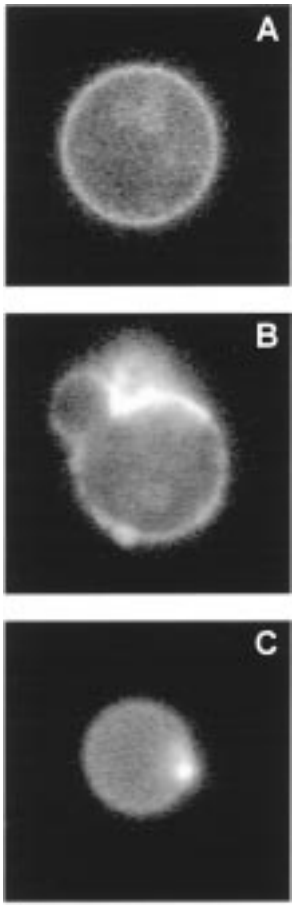

FIGURE 5: Domain formation by C1q visualized with NBD/PG. Micrographs visualizing NBD/PG fluorescence of an untreated giant liposome (A) and liposomes after the interaction with C1q (B) or the positively charged C1q fragment $C1qA_{(14-26)}$ (C) in barbital buffer, 20 mM NaCl, pH 4, are shown. The pictures were taken on the Nikon microscope approximately 2 min after mixing of giant liposomes with C1q or $C1qA_{(14-26)}$. The final concentrations in 10 μL of the mixture were 85 μM total lipid and 36 nM C1q or 140 μM $C1qA_{(14-26)}$, respectively. The structural changes led to bleb formation and accumulation of negatively charged lipid especially at the seam. The size range of the liposomes in this figure is 5−9 μm diameter.

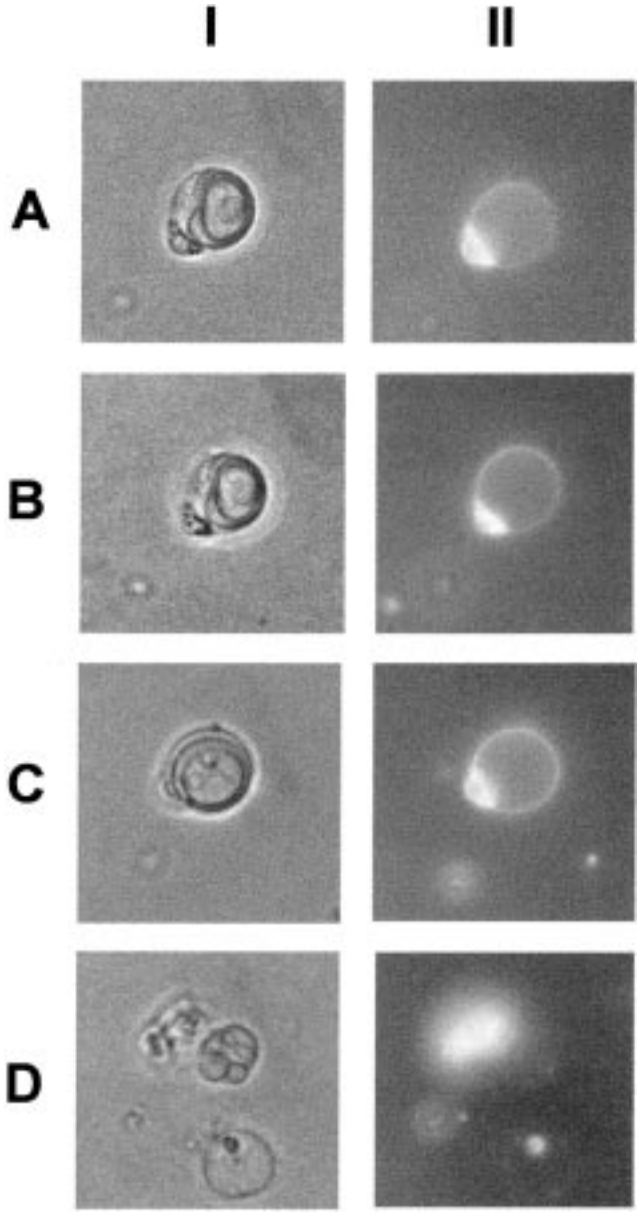

FIGURE 6: Domain formation by C1q visualized with Alexa 488-labeled C1q. A time series is depicted of the interaction of Alexa-488-labeled C1q and NBD/PG-labeled giant liposomes. The same liposomes were viewed in parallel with both phase contrast (I) and fluorescence microscopy (II) at pH 4 and 20 mM NaCl. One microliter of Alexa-488-labeled C1q (1 μM) was added to 9 μL of giant liposomes (96 μM total lipid) in the temperature-controlled well of the Zeiss microscope stage. The time between the frames A, B, and C was about 1 min each, and approximately 5 min elapsed between frames C and D. Shown in panels II is an accumulation of high Alexa-488 fluorescence on C1q that coincides with the bulging of the membrane seen in phase contrast microscopy, which was demonstrated to correlate with PG domain formation. Eventually the C1q-coated PG-containing membrane buds off. In panel D-II the upper liposome fragment stained with C1q-Alexa-488 is not well-resolved because it went out of focus. The size of the liposome shown is ~7 μm in diameter.

It is evident from the results of the studies reported here that electrostatic interactions are critical for binding to occur. First, the lack of C1q binding to neutral PC/CH (55:45 mol %) liposomes, under varied conditions of pH and ionic strength, supports the requirement for negative lipid surface charge. The pH, ionic strength, and anionic lipid composition dependence are also consistent with an electrostatic binding determinant. Maximal C1q binding occurred at a pH 4 (Figure 1) where C1q would carry a positive net charge. CL20 liposomes bound 85 times more C1q at saturation at pH 4.0 than at pH 7.2 (Table 1 and ref *23*); CL40 liposomes bound 15 times more C1q under the same conditions. C1q binding to PG40 liposomes was approximately the same as the C1q binding to CL20 liposomes at saturation ($5.3-6.0 \times 10^{-4}$ μmol of C1q/μmol of exposed lipid), consistent with the approximately equal net surface charge densities expected due to the charge of −2 per CL molecule (Table 1). Also, as the proportion of anionic phospholipid in the liposomes was increased, saturation binding at pH 4 likewise increased. Finally, in a previous study of the inhibition of C1q binding to liposomes by C1q-related peptides a net positive charge on the peptide was found to be critical for activity (*23*).

The literature contains other examples of C1q binding to negatively charged materials. C1q binding to dsDNA at low pH (pH 4.45) has been demonstrated. The study by Uwatoko and Mannik showed that while C1q binding to DNA occurred at low pH, these low pH conditions abolished C1q binding to heat-aggregated human IgG (*7*). This observation sug-

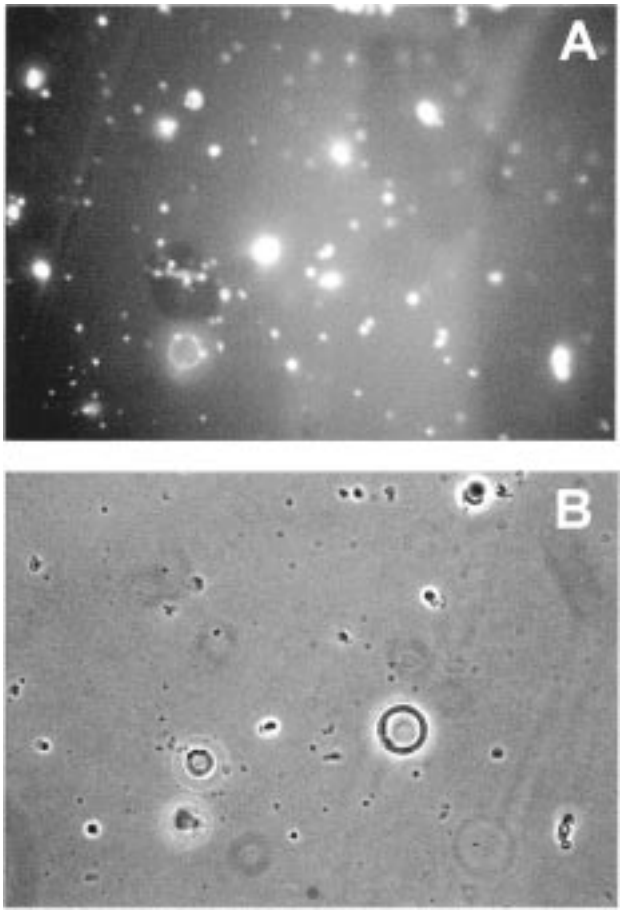

FIGURE 7: Domain formation by C1q visualized five minutes after the addition of alexa 488-labeled C1q. Fluorescence (A) and phase contrast (B) micrographs demonstrate the interaction of giant liposomes with Alexa-488-labeled C1q in barbital-buffered 20 mM NaCl, pH 4, approximately 5 min after liposomes and C1q were mixed (1 nM C1q/μmol of lipid). Many small, bright vesicles composed of C1q-coated PG-containing membrane stained with Alexa-488 have pinched off from large vesicles. The largest vesicle seen in the phase contrast image is $\sim$7 μm in diameter.

gested that a conformational change in C1q may occur at low pH which affects the globular headgroups of the protein (the IgG binding sites) but not the collagen-like region which is involved in DNA binding. Our observation of the reversibility of the effect of exposure to low pH on C1q binding to anionic liposomes is consistent with this report.

The effect of pH on protein binding to anionic liposomes has previously been reported by Bergers et al. (*37*), who studied the adsorption of a number of proteins, including myoglobin which has an isoelectric point (IEP) similar to that of C1q ($IEP_{myoglobin}$ = 6.9−7.4) (*34*), to anionic liposomes as a function of pH at low ionic strength (I = 0.01 M). These investigators found that adsorption of proteins to anionic liposomes was only measurable at pH values for which the proteins had an overall positive charge. The amount bound at 1 mg/mL protein depended on the pH of the incubation medium and correlated with the magnitude of the net positive charge of the protein.

Another indicator of the central role of electrostatic interactions in C1q/liposome binding is the ionic strength dependence observed. C1q binding to anionic extruded liposomes and MLVs was enhanced enormously when the ionic strength was lowered from physiologic (0.145 M) to 0.02 M (Figure 3); saturation binding to 240 nm CL20 liposomes increased by over 200-fold in this range (Table 1).

Our data on the ionic strength dependence are in agreement with a number of studies investigating the nature of C1q binding to other substances such as fibrinogen, fibrin (*38*), fibronectin (*39*), collagen (*24*), and bacterial lipopolysaccharides (*40*). These studies measured C1q binding under subphysiologic ionic strength conditions (0.015−0.07 M). Even C1q binding to specific receptors on cells and platelets was found to be ionic strength-dependent. For instance, Peerschke and Ghebrehiwet (*41*) showed enhanced adhesion of C1q to platelet receptors at 20 mM NaCl compared to 150 mM. The pH and ionic strength data in this paper also confirm and quantitate previous observations in our laboratory using particle electrophoresis to monitor C1q/CL20 liposome interactions at pH 5 and pH 7 under different ionic strength conditions (*23*).

In analyzing the binding data we have utilized the simplest equation that provides satisfactory fits (eq 1). This equation results from the analysis of any equilibrium reaction in which two species, one of which is limited in amount, interact reversibly by mass action to form a product. The equation is of the same form as the Langmuir isotherm and can be transformed to give the Scatchard equation (*42*), but its use here does not imply the assumptions inherent in either model. In particular, we do not assume that the apparent association constant necessarily represents a chemical equilibrium constant that describes the standard-state free-energy change associated with a single association event, although this possibility is not excluded. Equation 1 is also used widely to describe the nonspecific adsorption of proteins to surfaces (*43*). In this case it is recognized that the apparent association constant represents the net effect of a potentially large number of individual associations of specific groups on the protein with the surface. Nonetheless, its magnitude is a measure of the affinity of the adsorbing protein for the surface, systems with higher values of K_a showing more adsorption at a low concentration than systems of the same binding capacity for which K_a is smaller.

The most remarkable feature of our results is that the apparent association constant is virtually independent of the magnitude of the electrostatic interaction between C1q and the lipid surface. The data in Table 1 show that K_a is essentially independent of pH, ionic strength, and anionic lipid composition, despite large variations in the saturation binding as conditions are changed. For 12 different conditions over which the above parameters were varied, the average value for K_a was $3.7 \pm 2.3 \times 10^8$ (M^{-1}). Only two values fell outside this range and both were lower than the average by less than 1 order of magnitude.

There is now a considerable literature on the association of positively charged peptides with negatively charged lipid surfaces (*44*−*48*), partly because of interest in the role such interactions play in signal transduction pathways, particularly those involving protein kinase C (PKC) and basic peptides derived from one of its substrates, MARCKS (Myristolated Alanine-Rich C Kinase Substrate) (*49*−*51*). A large body of data is consistent with a model in which many small basic peptides, bearing typically 2−5 positive charges at neutral pH, associate with negatively charged lipid surfaces by accumulating in the electrical double layer but do not interact

via chemical bonds with the lipid molecules. That is, they are associated with the surface by electrostatic interactions but depend weakly if at all on nonelectrostatic factors.

This kind of model has been applied to larger polypeptides of the α-K-toxin family (∼4000 Da) with some success as well (*47*). A characteristic feature of such association is the very strong dependence of the apparent association constant or partition constant (i.e., the ratio of bound to free peptide) on the electrostatic free energy of the system. For instance (*47*), the partition coefficient *K* for charybdotoxin (CTx) associating with 2:1 PC/PS vesicles decreased by a factor of 50 000 when the ionic strength was increased from 10 to 200 mM. Likewise *K* increased by a factor of about 10 when the acidic lipid concentration in the membrane was increased from 18 to about 36 mol %. All of the measurements on which these calculations are based were carried out at very low peptide concentrations, however, where the total amount of peptide in the system was much less than the amount of lipid available. Hence, no saturation effects were observed. Nonetheless, the difference between the results in Table 1, in which K_a was found to be independent of electrostatic factors, and those observed for basic peptides is striking.

Others have observed the effects of binding saturation for oligo- and polylysine (*45*) on pure PG membranes, but no apparent association constants were reported and the electrostatic factors were largely held constant. Binding of proteins to lipid vesicles has been studied by electrophoresis combined with uptake measurements of radiolabeled protein (*37*, *52*). In both studies it was found that factors that increased electrostatic attraction between lipid and protein enhanced binding. However, some lipid specificity was found in the study of a PKC isoform binding to negatively charged lipid vesicles, implying that chemical interactions between protein and lipid were partially involved in determining binding behavior (*52*). The study by Bergers et al. (*37*) was largely based on electrophoretic mobility measurements of lipids equilibrated with pure proteins. This approach does not allow calculation of association constants or saturation binding levels for such complex systems unless very restrictive assumptions are made. However, for several proteins the effects on lipid vesicle mobility of protein exposure at a series of solution pH values were found not to be readily reversible when the suspending medium was diluted 10-fold with buffer, implying that direct protein-surface associations were involved. This whole study was carried out at an ionic strength of 10 mM, however, which would tend to maximize any direct ± charge interactions.

The saturation binding values we observed indicated that under conditions of strong electrostatic interactions the surface of the 240 nm liposomes was probably saturated. Using the maximum estimated area per molecule quoted above, a 240 nm vesicle could carry ∼150 molecules per vesicle if the molecules occupied their maximum cross-section. Many of the saturation values in Table 1 are near this value. The molecule has a usual shape, however, somewhat analogous to a beach umbrella. The available evidence is that binding to polyanionic sites occurs near the end of the handle of the beach umbrella. More beach umbrellas can be accommodated per unit area if the umbrella is lying on its side with the handles in contact laterally with the surface; the recognized flexibility of the hinge region would allow this (*35*). Hence, the packing achievable could be considerably higher than ∼150 per vesicle. It is also possible that some aggregation of C1q contributes to the high saturation values, although this would not be expected at high ionic strength where in fact our largest values were obtained (for CL40 at pH 4).

Formation of Lipid and Protein Domains. It is known that the association of anionic lipid-containing membranes with divalent cations (*33*), basic proteins (*53*), and basic peptides (*49*−*51*, *54*) can lead to the formation of domains enriched in the acidic lipid. A simple model for the effect of basic peptides has been proposed, in which the reduction in electrostatic free energy associated with accumulation of positive charges near the negative lipid is sufficient to overcome the unfavorable decrease in the entropy of mixing that occurs when the lipid is concentrated locally (*54*). This model, which considers only the two types of interactions just mentioned and does not deal with effects associated with domain boundaries, predicts that as the peptide concentration is increased domain formation goes through a maximum but at high amounts of peptide, binding occurs on both the domain and nondomain surfaces, making the electrostatic potential more spatially uniform. Fluorescence images of LUVs containing acidic lipids and exposed to basic peptides, in which independent fluorescent probes were used to label negative lipids and peptides, showed domain formation induced by the MARCKS basic peptide, pentalysine, and a tetravalent spermine peptide. In the latter two cases, no localized fluorescence was observed at high peptide concentrations, supporting the predictions of the model.

In our studies domain formation was also observed (Figures 4−7). Poly-DL-lysine, C1q, and the basic $C1q_{(14-26)}$ peptide all induced domain formation within a minute of exposure to PG40 LUVs. In all cases the peptide or protein colocalized with the anionic lipid. In general the domains initially formed regions of higher radius of curvature than that of the body of the LUV, which pinched off into bright fluorescent vesicles leaving large or collapsed vesicles with little or no detectable fluorescence (Figures 6 and 7). This general sequence of events was observed with all anionic lipid compositions and solution conditions that produced enhanced saturation binding of C1q when compared to suspending media at pH 7/145 mM NaCl.

Role of Line Tension in Vesiculation. The determinant of the shape of lipid vesicles has been the subject of much recent discussion (*55*−*58*). In general, vesicles will tend to take up a closed form to minimize contact of any lipid edges with water, the shape being determined by the relative area of the inner and outer leaflets, the area-to-volume ratio of the membrane to the interior, any shape differences between lipids on the inner and outer leaflets of the bilayer, and the lateral distribution of lipid molecules in the plane of the membrane.

The formation of domains can have a significant effect on the local membrane shape (*57*, *59*). The shape taken up by the domain will be determined by the balance between the bending energy of the domain, which is independent of domain size, and the tension that develops at the boundary between the domain and the surrounding lipid (*57*, *59*). This tension is known as the line tension or edge energy. It is directly analogous to the interfacial tension exhibited by the two-dimensional surface which separates two bulk phases, such as oil and water or air and water. When the two phases,

or domains, are two-dimensional, the dividing "surface" is a line and the tension is the line tension, with units of energy per length. Physically, it derives from the asymmetry in nearest neighbor interactions across the boundary. Increasing the domain size therefore costs energy in proportion to the length of the domain boundary. The bending energy, on the other hand, is independent of domain size (*60*). The surface therefore can reduce its energy by forming a bud with the domain boundary at its base, since in this way the periphery will reduce in length, reducing the edge energy of the domain. Provided that the line tension times the boundary length is equal to or greater than the bending energy, the bud will tend to pinch off to form a vesicle. The critical diameter at which this will occur for phospholipid vesicles has been estimated to be about 4 μm (*57*). The predicted structures have been previously observed for thermally stressed liposomes (*55*). Domains in smaller vesicles will still experience an edge energy which will increase with the length of the boundary, but it is predicted to be too small to overcome the bending rigidity of the membrane. Hence, vesiculation would not be expected to occur in the smaller liposomes such as those used in most of our binding measurements.

The magnitude of the line tension for phospholipid domains has been estimated to be $\sim 10^{-12}$ J/m (*61*) or, at room temperature, $\sim$ 0.25 kT/nm. Hence, for a domain the size of the maximum area per molecule estimated for C1q, $(35\ \text{nm})^2$, the circumference would be $\sim 35\pi$ nm and the energy required to create the boundary would be of the order of 27 kT. While this value must be considered an upper limit, it indicates that the effect of line tension can be very significant. Certainly it would provide an additional driving force for domain formation since the total periphery of the domain would be minimized to minimize the total edge energy. Therefore, there will be a strong tendency for any individual domains to fuse into a single region with minimum circumference.

If this value is much smaller than the standard-state free-energy change associated with the binding of C1q, the line tension effect on binding would be expected to be small. However, estimating this free-energy change as $RT \ln K_a$ gives a value of $\sim$20 kT per molecule, of the same order of magnitude. Hence, it seems quite possible that the edge energy could have a significant effect on the standard-state free-energy change associated with C1q binding in this system.

A Model for the Independence of K_a from Electrostatic Factors. On the basis of the above discussion, we propose a qualitative model for the lack of dependence of the apparent association constant on electrostatic factors. C1q binding in these simple systems requires anionic lipids. Association of C1q encourages the formation of local domains of anionic lipid via electrostatic attraction between the clusters of positive charges on C1q, possibly in the region of residues 14−26, and the negative lipid, as discussed for a related system by Denisov et al. (*54*). The overall electrostatic effect increases as the anionic lipid content or total protein positive charge is increased or the ionic strength is decreased, creating larger domains and allowing more C1q to bind. The increasing domain size results in concomitant increases in the domain edge energy, however, which act to reduce the overall binding energy despite the greater electrostatic interaction responsible for the saturation binding increases. The net effect is that K_a remains essentially unchanged as conditions are varied to enhance electrostatic interactions.

It is possible to estimate the approximate dependence of the edge energy on the number of lipid molecules in a domain, which is presumably a reflection of the amount of protein bound as well. Because the edge energy is proportional to the circumference of the domain, it will vary as $N^{1/2}$ where N is the number of either anionic lipid or C1q molecules per domain. The free-energy change per bound molecule will be affected by the electrostatic interaction energy as well, but this increases in magnitude relatively slowly as the surface charge is increased or the ionic strength decreased (*47*). Hence, the edge energy may not be overwhelmed by the electrostatic component as the domain sizes grow. The net energy change will be determined by the balance between the two, with the result that the apparent binding constant can remain essentially invariant, as observed.

While we have not attempted a quantitative test of the above model, our data and observations are consistent with its predictions. Apart from the constancy of K_a, the saturation binding data indicate that a finite number of binding sites are available on each vesicle that is determined by the anionic lipid composition and the electrostatic energy in the system. Clearly the liposome surface is not saturated because of geometric packing constraints at pH 7/145mM NaCl, consistent with the formation of small domains that grow as the anionic lipid composition is increased. The time course of binding measured ($\sim$min) parallels the time courses of anionic and protein domain formation observed microscopically. The very obvious kinetics and presence of domain formation under conditions of strong electrostatic interactions clearly demonstrate that binding takes place predominantly in these accumulations of anionic lipids.

The geometry of the domains, with their altered radius of curvature, is also consistent with the model. The fact that the domains appear circular, such as in Figures 4C and 5C, supports the presence of a significant line tension, since this shape minimizes the circumference. Nonspherical shapes are also possible; they would indicate that the system was near the critical conditions for formation of the domain phase and the line tension lower (*61*). The presence of bulged domains (Figures 4 and 6) is also consistent with line tension effects. The boundary of such a bulged region is shorter in length than if the domains were flat. There are other explanations, however, in that the curvature could be associated with differences in bending rigidity between the anion-rich and -poor domains (*60*). The curvature could also be associated with electrostatic effects (*62*).

The subsequent fate of the bulges, which bud, form vesicles, and either pinch off into suspension or adhere to the liposome surface and carry virtually all of the fluorescence with them, is precisely described by the anticipated line tension effect. The body of the vesicle left behind (Figures 6 and 7) is generally devoid of visible C1q fluorescence, consistent with the loss of the domains to small vesicles.

There is also some sign of this behavior in the binding data. When 240 nm vesicles, too small to vesiculate, were used as substrate for binding, the saturation ratio of C1q to total lipid was never greater than $\sim 5 \times 10^{-4}$ at low ionic

strength (obtained by multiplying the values in Table 1 by the fraction of exposed lipid). When MLVs were used, which are greater than 4 μm diameter so that vesiculation would be expected, saturation appeared to increase by an order of magnitude at low ionic strength when calculated on the basis of exposed lipid (Table 1). We have observed fluorescently labeled multilayered "LUVs" under the same conditions and observed, after loss of bright vesicles, breakdown of internal layers and further vesiculation into brightly fluorescent spots. Hence, in multilayered MLVs vesiculation may result in more than just the outer bilayer being exposed to binding protein, with subsequent recruitment into domains of anionic lipid originally internalized in the liposomes. When the saturation binding at low ionic strength is calculated per total lipid, the value is $\sim 6 \times 10^{-4}$, essentially indistinguishable from the value for 240 nm vesicles. Thus, the MLV values suggest that C1q has access to the same fraction of lipid present as in the smaller liposomes. Vesiculation would provide a mechanism whereby this might occur. Hence, pinching off of domains due to edge energy minimization can rationalize the very high apparent MLV saturation binding values observed.

SUMMARY AND CONCLUSIONS

Consideration of the line tension effects associated with domain formation provides a rationale for our binding data and microscopic observation of domain formation and its subsequent vesiculation. The qualitative model we have proposed can be made considerably more quantitative and it should, perhaps in a simpler system, be possible to test such a model experimentally. We believe such an effort to be worthwhile since there are a number of interesting ramifications which follow if these ideas can be verified.

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