ELSEVIER

Biochimica et Biophysica Acta 1418 (1999) 19–30

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

C1q Binding to liposomes is surface charge dependent and is inhibited by peptides consisting of residues 14–26 of the human C1qA chain in a sequence independent manner

Amanda J. Bradley [a], Donald E. Brooks [a,b], Raymond Norris-Jones [a], Dana V. Devine [a,c,*]

[a] *Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, BC V6T 2B5, Canada*

[b] *Department of Chemistry, University of British Columbia, Vancouver, BC, Canada*

[c] *Canadian Blood Services, Vancouver, BC, Canada*

Received 31 August 1998; received in revised form 7 January 1999; accepted 11 January 1999

Abstract

Complement activation by anionic liposomes proceeds by antibody-independent, C1q-initiated activation of the classical pathway. Purified C1q bound to anionic liposomes in an acidic lipid concentration-dependent manner. Saturation binding, but not the apparent association constant, was enhanced by increasing the cardiolipin content of the liposomes or decreasing either the pH or ionic strength of the reaction mixture. These observations indicate the involvement of electrostatic factors in the binding. A highly cationic region in the collagen-like domain of C1q comprised of residues 14–26 of the C1qA polypeptide chain was assessed for involvement in liposome binding. This region has previously been shown to mediate C1q binding to other immunoglobulin-independent activators of the classical pathway of complement. Peptides containing residues 14–26 of C1qA, denoted $C1qA_{14-26}$, inhibited C1q binding to and complement activation by anionic liposomes. The inhibitory capacity of these cationic peptides had no sequence or conformation specificity. Rather, the amount of positive charge on the peptides was the determining factor. When present in excess, peptides with five cationic residues inhibited C1q binding and complement activation; however, C1q peptides with only two cationic residues did not. In addition to the $C1qA_{14-26}$ region, other parts of C1q that contain cationic residues may also be involved in C1q binding to anionic liposomes.

Keywords: Liposome-mediated complement activation; C1q binding; Liposome clearance

1. Introduction

Liposomes are used for a variety of applications from serving as model membranes to the delivery of therapeutic agents. A major limitation to the in vivo use of liposomes is the fact that in general, injected liposomes are eliminated from the circulation by the reticuloendothelial system. Over 90% of neutral liposomes end up in the liver within a few hours of

Abbreviations: CH, cholesterol; CL, cardiolipin derived from bovine heart; CL20, liposomes composed of EPC/CH/CL at 35:45:20 mol%; EPC, phosphatidylcholine (PC) derived from egg; GVB^{2+}, gelatin veronal-buffered saline with 0.15 mM Ca^{2+} and 1 mM Mg^{2+}; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; VBS, veronal-buffered saline

* Corresponding author. Fax: +1-604-822-7635;
E-mail: ddevine@pathology.ubc.ca

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00013-9

injection [1] and certain negatively charged liposomes are cleared even more rapidly, with half-lives in the circulation on the order of a few minutes [2]. The mechanism for the efficient uptake of injected liposomes is believed to involve association with and opsonization by plasma proteins [3].

Complement proteins are amongst the gamut of immunoproteins responsible for opsonization and clearance of foreign particles from the blood. Many investigators have shown that liposomes can activate complement, depending on their composition and physical features (reviewed in [4]). The activation of complement by liposomes is an important issue as it can result in loss of liposome stability with subsequent leakage of entrapped substances [5,6] or liposome opsonization by C3b or iC3b leading to enhanced uptake by phagocytic cells [7–12]. Complement activation also results in the release of fragments C3a and C5a. The chemotactic behavior of these fragments allows for signaling of phagocytic cells; Scieszka et al. [8] demonstrated that the generation of C5a enhanced PMN phagocytosis of liposomes.

In addition to antibody-mediated complement activation, anionic liposomes can also initiate complement in the absence of immunoglobulins, most likely through the direct binding of C1q [13]. C1q is an unusually shaped, 465 kDa glycoprotein that consists of six globular headgroups connected by a triple helix collagen-like stalk [14]. Their are six copies each of the three distinct polypeptide chains, A, B and C. The triple helices of the collagen-like region begin close to the N-terminus of each polypeptide chain and continue to about residue 89. The remaining ~131 residues of each chain fold to form the globular head domains [15]. 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 [16]. In the absence of antibody, C1q binds directly to a variety of substances, including a number of different proteins, polyanions, cell structures, DNA, and many different cell types including platelets. A group of antibody-independent complement activators including serum amyloid P (SAP) [17], C-reactive protein (CRP) [18], DNA [19], and β-amyloid fibers [20] has been shown to bind the collagenous region of C1q. These activators share a common property: they all have repeating negative charges. The specific region of the collagenous stalk of C1q that is believed to mediate binding to this group of activators is contained within the highly cationic region of residues 14–26 of the C1qA polypeptide chain [17–20]. Because anionic liposomes have repeating negative surface charges, the possibility that the $C1qA_{14–26}$ region of C1q may play a role in mediating C1q–liposome interactions was assessed in the present study. The nature of the interaction between C1q and anionic phospholipids was also investigated.

2. Materials and methods

Unless otherwise noted, all chemicals were purchased from Sigma Chemicals (St. Louis, MO).

2.1. Purification and radiolabeling of human C1q

C1q was purified from fresh acid citrate dextrose-anticoagulated human plasma obtained from the Canadian Red Cross Society Blood Services using the method of Tenner et al. [21] with the following modifications. Prior to chromatography on BioRex 70 (BioRad, Hercules, CA), serum lipids were removed by centrifugation at $12\,500 \times g$ for 30 min at 4°C. To increase retention of C1q on the column, the BioRex column was run at pH 7.1 rather than pH 7.3. Fractions containing functional C1q were detected by a C1q ELISA which measures C1q bound to human IgG-coated wells [13]. Purified C1q was stored in 50 mM Tris with 0.5 M NaCl at −80°C in 50-μl aliquots and was used without repeated freeze–thaw for up to 1 year from the isolation date. C1q was radiolabeled using the Iodogen method (Pierce Chemicals, Rockford, IL). The molar ratio of $Na^{125}I$ (Amersham) to C1q was either 1:4 or 1:10, depending on the specific activity desired.

2.2. Peptides

C1qA peptides were synthesized by the Biotechnology Protein Service Laboratory, University of British Columbia. The following peptides were made: authentic $C1qA_{(14–26)}$ (A-G-R-P-G-R-R-G-R-P-G-L-K); $C1qA_{(scrambled)}$ (K-P-R-G-L-G-G-A-G-R-R-P-R); $C1qA_{(P–A)}$ in which both proline residues were

replaced with alanine residues (A-G-R-A-G-R-R-G-R-A-G-L-K); C1qA$_{(2+)}$ in which three cationic residues were replaced with glycine residues (A-G-G-P-G-R-G-G-R-P-G-L-G); and C1qA$_{(0+)}$ in which all cationic residues were replaced with glycine residues (A-G-G-P-G-G-G-G-G-P-G-L-G). A 13-amino-acid peptide from the terminal sequence of fibrinogen, (Fgn) (C-H-H-L-G-G-A-K-Q-A-G-N-V), was used as a control.

2.3. Preparation of liposomes

Liposomes were made as previously described [22]. Multilamellar vesicles (MLVs) were extruded under pressure through polycarbonate filters (Costar, Cambridge, MA) (extruder from Lipex Biomembranes, Vancouver, Canada). Large unilamellar vesicle (LUV) size distributions were analyzed using the Nicomp Submicron Particle Sizer (Model 270) (Particle Sizing Systems, Santa Barbara, CA). Liposomes extruded through 100-nm pore size filters were 110 ± 20 nm in diameter; liposomes extruded through 400-nm pore size filters were 240 ± 38 nm in diameter. The total lipid concentration was determined from the concentration of phosphate [23] and the molar ratio of phosphate to the phospholipids. Phosphatidylcholine (EPC) and cardiolipin (CL) were purchased from Avanti Polar Lipids (Alabaster, AL). The liposome composition was EPC/CH/CL at ($35-n$:45:n mol%). These studies have mainly focused on compositions containing 20 mol% anionic phospholipid, as we have previously reported [24] that there is a significant increase in complement activation by compositions containing 20 mol% compared to 10 mol%.

2.4. Equilibrium C1q binding assay

Equilibrium binding measurements were made with liposomes suspended in buffer (1.8 mM sodium barbital, 3.1 mM barbituric acid, 11% sucrose) with pure C1q; NaCl concentrations varied from 20 to 145 mM. Reaction mixtures consisted of 15 μl of C1q/[^{125}I]C1q mixture, 5 μl of sucrose-containing $\sim$240 nm liposomes, and 80 μl of diluting buffer. Liposomes of this size were used in order to achieve effective separation by the centrifugation method described below. To obtain a final ionic strength of 145 mM NaCl, the dilution buffer consisted of 1.8 mM sodium barbital, 3.1 mM barbituric acid, 77.9 mM NaCl, and 7.2% D-glucose. Following a 20-min reaction at room temperature (RT) (results were time-independent for 1 to 60 min), 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 145 mM NaCl) in 5×20 mm polyallomer tubes. Tubes were centrifuged for 30 min at $166\,300 \times g$ to pellet $>90\%$ of the liposomes, frozen, then sliced into two pieces: a pellet slice containing liposome-bound C1q and a supernatant slice. Tube slices were counted in an LKB Wallac gamma counter (Compugamma model 1282). Tubes containing C1q but no liposomes were run in parallel (C1q control). The amount of [^{125}I]C1q spun down in this control tube was always less than 8% and was subtracted from the binding values for the liposome/C1q reaction tubes. The amount of C1q bound to liposomes was calculated as follows:

$$\mu g\ C1q\ bound = (cpm_{pellet} - cpm_{C1qcontrol}) / \text{specific activity of C1q mixture} \quad (1)$$

2.5. Inhibition of C1q binding to anionic liposomes by peptides

To measure the inhibition of C1q binding to liposomes by C1qA peptides, peptides were mixed with liposomes and [^{125}I]C1q in buffer and binding was measured as in Section 2.4. Reaction mixtures consisted of 74 μl diluting buffer, 15 μl of C1q/[^{125}I]C1q mixture, 5 μl of sucrose-containing 240-nm liposomes, and 6 μl of peptide or peptide buffer. The percent inhibition of C1q binding was calculated as follows:

$$\%\ inhibition = 100 - (C1q_{bound(CL20lipo+peptide)} / C1q_{bound(CL20)} \times 100) \quad (2)$$

where $C1q_{bound} = (\%\ of\ cpm\ in\ pellet)_{sample} - (\%\ of\ cpm\ in\ pellet)_{C1q\ control}$

2.6. Functional complement assay

The capacity of C1q peptides to inhibit complement activation by anionic liposomes was measured

in a modified hemolytic assay. Liposomes (~100 nm in diameter) were suspended in isotonic veronal-buffered saline (VBS: 1.8 mM sodium barbital, 3.1 mM barbituric acid, 145 mM NaCl, pH 7.4). These studies were performed using 100-nm vesicles since extruded liposomes of this size and composition have little or no multilamellar character, allowing better estimates of exposed surface area. Normal human serum (NHS) was prepared from venous blood collected from healthy donors and stored at −80°C. Peptides were first serially diluted in VBS containing 0.15 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% gelatin (GVB^{2+}). An equal volume of EPC/CH/CL (35:45:20 mol%) liposomes (5 mM) was added to the peptides and the mixture was incubated at RT for 40 min. An equal volume of NHS diluted 1:4 in GVB^{2+} was then added to each tube. After 30 min at 37°C, three volumes of GVB^{2+} were added to the serum/liposome mixture. Controls consisted of a color blank (liposomes incubated with buffer and without serum), a 100% lysis control (serum incubated in the absence of liposomes or peptides), a CL20 liposome control (liposomes with serum and no peptides), and peptide controls (peptides with serum and no liposomes).

The total residual complement content of the liposome-treated serum was then measured by adding antibody sensitized sheep red blood cells (EA cells) to the liposome/serum mixture as previously described [13]. The level of EA lysis for CL20 liposome control tubes was only slightly higher than the EA lysis in the color blanks indicating that virtually all of the complement in these tubes was activated by the liposomes in the absence of peptides. The percent inhibition of complement activation was equivalent to the percent EA lysis and was calculated as follows:

$$\%\ \text{inhibition of activation} = \frac{(A_{415\ \text{for test sample}} - A_{\text{color blank}})}{(A_{415\ \text{for 100\%}} - A_{\text{color blank}})} \times 100 \quad (3)$$

2.7. Particle electrophoresis

Particle electrophoresis permits direct microscopic observation of the behavior of individual liposomes in an electric field by the use of a water immersion objective and a quartz cylindrical electrophoresis chamber (Rank Mark I, Rank Bros, Bottisham, UK). The method may be used to provide a direct calculation of the zeta potential without the artifacts that may be introduced into other techniques such as laser Doppler by the particles themselves. In order to visualize the liposomes, this method requires the use of MLVs. To assess the interaction of C1q or C1q peptides with liposomes, MLVs at 0.36 mM total lipid in 1.8 mM sodium barbital, 3.1 mM barbituric acid, with either 20 mM, 100 mM, or 145 mM NaCl, pH 5.0 or 7.0, were pre-incubated with C1q or peptides for 40 min at RT in a total volume of 780 μl. Just prior to flooding the electrophoresis chamber with the reaction mixture, the same buffer was added to bring the mixture volume to 2.8 ml. C1q or C1q peptide binding to liposomes was then monitored as described previously [25]. Ten or more particles were timed per experimental condition as they moved across the eyepiece reticule. The mobility was calculated from the averaged velocities, the applied voltage, and the chamber electrical length according to the following calculation [26]:

$$\text{electrophoretic mobility}(\mu) = \frac{\text{particle velocity } (\mu\text{m/s})}{\text{electric field strength } (E)\ (\text{V/cm})} \quad (4)$$

where E = (voltage/l) and l = the effective distance between the electrodes.

3. Results

3.1. The effect of liposome surface charge density on C1q binding

Quantitative measurements of C1q binding to liposomes were made using the C1q equilibrium binding assay. No C1q binding to neutral EPC/CH (55:45 mol%) liposomes was detected. C1q binding to anionic liposomes depended upon the amount of anionic phospholipid. As the anionic phospholipid content increased, a concomitant increase in the amount of C1q binding to liposomes was observed (Fig. 1). While increasing cardiolipin from 20 mol%

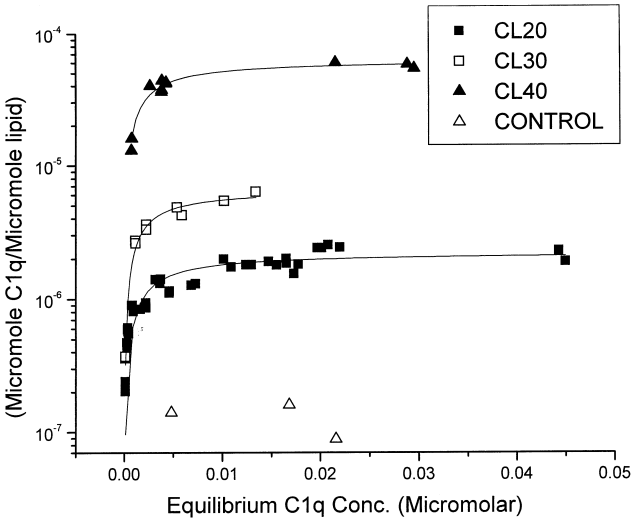

Fig. 1. C1q binding to cardiolipin-containing liposomes: effect of surface charge density. C1q binding to 240 nm liposomes at pH 7.2 was measured using the C1q equilibrium binding assay. Liposomes were composed of EPC/CH/CL at (55−*n*):45:*n* mol % where *n* = 0 (△), 20 (■), 30 (□), or 40 (▲) mol% of CL. The solid lines are plots of the equation given in the text utilizing the best fit values for the parameters given in Table 1. A constant total lipid concentration of 0.5 mM was used in the reaction. Because far greater levels of C1q binding were measured for CL40 liposomes, the vertical axis is presented in log scale. Most data points for EPC/CH liposomes (△) were zero; these are not shown due to the log scale.

to 30 mol% resulted in a 4-fold increase in the amount of C1q binding at saturation, doubling the cardiolipin content resulted in 40-times more C1q binding (Table 1). Each data set was fit to the fundamental binding (Langmuir) equation:

$$Y = (P1\ P2\ X)/(1 + P2\ X) \quad (5)$$

where Y = amount of C1q bound/μmol of lipid, $P1$ = saturation level of binding, $P2$ = association constant (1/M) and X = C1q concentration (M). Data sets were fit utilizing the non-linear least squares procedure in Origin (Microcal). Fig. 1 illustrates the data (points) and the best fit curves (solid lines). The parameters of best fit for each set are given in Table 1. In all cases the data fit the above equation satisfactorily over the whole range of concentration examined.

3.2. Effect of ionic strength and pH on C1q–liposome binding

Since the importance of electrostatics was implied by the dependence of C1q binding on liposome surface charge, the effects of ionic strength and of pH on C1q binding were investigated. C1q–CL20 liposome interactions were monitored at pH 5.0 and pH 7.0 by particle electrophoresis under different ionic strength conditions (Fig. 2). It is important to note that the electrophoretic mobilities reflect the extent of C1q–liposome interactions, but the method does not allow for quantitative interpretation of the amount of C1q bound to the liposomes. The mobilities of MLVs without C1q decreased as the ionic strength went from 20 mM NaCl up to 145 mM, as expected. Liposome mobilities were identical at pH 5 and pH 7. When C1q was reacted with CL20 MLVs, the mobility shifted towards zero as C1q binding to MLVs decreased the liposome surface charge. The magnitude of the liposome mobility shift after incubation with C1q was inversely related to ionic strength; CL20 MLVs bound more C1q at lower

Table 1
Apparent association constants and saturation values for C1q binding to liposomes: effect of liposome surface charge density

Liposome composition	χ^2 fit to equation	K_a (M^{-1}) ± E.S.E.	Saturation binding	
			μmol $C1q_{bound}$/μmol total lipid ± E.S.E.	molecules of $C1q_{bound}$/vesicle
EPC/CH/CL (35:45:20)	6.4×10^{-14}	$4.5\times10^{8}\pm0.8\times10^{8}$	$2.2\times10^{-6}\pm9.8\times10^{-8}$	3/2
EPC/CH/CL (25:45:30)	1.3×10^{-13}	$5.1\times10^{8}\pm1.0\times10^{8}$	$6.6\times10^{-6}\pm4.0\times10^{-7}$	5/1
EPC/CH/CL (15:45:40)	1.0×10^{-11}	$4.7\times10^{8}\pm0.6\times10^{8}$	$6.0\times10^{-5}\pm2.3\times10^{-6}$	50/1

Saturation binding and association constants for CL20, CL30 and CL40 liposomes were derived from the non-linear least-squares fit and are reported with the estimate of standard error (E.S.E.). To convert μmol total lipid to number of vesicles, the surface area per vesicle and the number of vesicles per μmol total lipid were first estimated. The surface area per vesicle was calculated as $SA_{vesicle} = 4\pi r^2$ and was 1.8×10^{-9} cm^2/vesicle for our system. The number of 240 nm vesicles per μmol total lipid was estimated to be 8.0×10^{11} vesicles/μmol lipid, assuming that 35% of the total lipid is exposed [28] and that the average area per lipid is 0.7 nm^2.

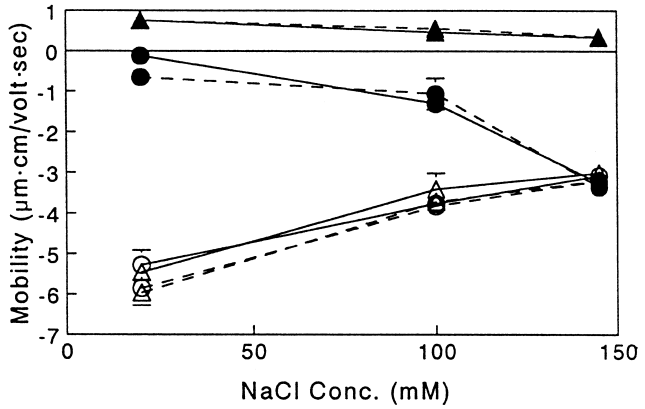

Fig. 2. Ionic strength and pH dependence of C1q binding to CL20 MLVs. Particle electrophoresis was carried out on CL20 MLVs alone (open symbols) or in the presence of C1q (closed symbols) under different NaCl concentration conditions at pH 5 (△,▲) and 7 (○,●). Where liposomes were preincubated with C1q, the total lipid in the reaction was 0.36 mM and the C1q concentration was 0.134 mM (61.5 μg/ml). The complete experiment was carried out twice; one data set is shown by the solid lines and the other by the dotted lines. Error bars represent one standard deviation of the mean of the mobilities of the ten vesicles. Where no error bars are shown, one S.D. was smaller than the size of the symbol used for the mean.

ionic strength. This trend was observed whether the pH was 5 or 7 but was more pronounced at pH 7. When C1q was added at pH 7, liposome mobilities decreased more than 5 mobility units at 20 mM NaCl but did not change at 145 mM NaCl. C1q had larger effects on the mobilities of CL20 MLVs at pH 5 than at pH 7 under every ionic strength condition tested.

While liposome mobilities were being measured, the aggregation state of the liposomes was monitored. Liposome aggregation was observed under conditions where C1q binding to anionic liposomes was enhanced, at low ionic strength (20 mM NaCl) and at low pH (pH 5.0). At pH 7 and 145 mM NaCl, no aggregation was observed.

3.3. *Inhibition of C1q binding to CL20 liposomes by C1qA peptides*

C1qA, C1qA-control, and unrelated peptides were examined for their ability to compete with C1q in the C1q equilibrium binding assay (Fig. 3A). At 240 μM, the $C1qA_{14–26}$ authentic peptide completely blocked CL20–C1q binding; at 50 μM, 70% of C1q binding was inhibited. C1qA control peptides, $C1qA_{(scrambled)}$ and $C1qA_{(P–A)}$, also inhibited C1q binding to CL20 liposomes suggesting that a peptide conformational or sequence specificity was not required. The charge control peptides, $C1qA_{(0+)}$ and $C1qA_{(+2)}$, did not inhibit C1q–CL20 binding, indicating that more

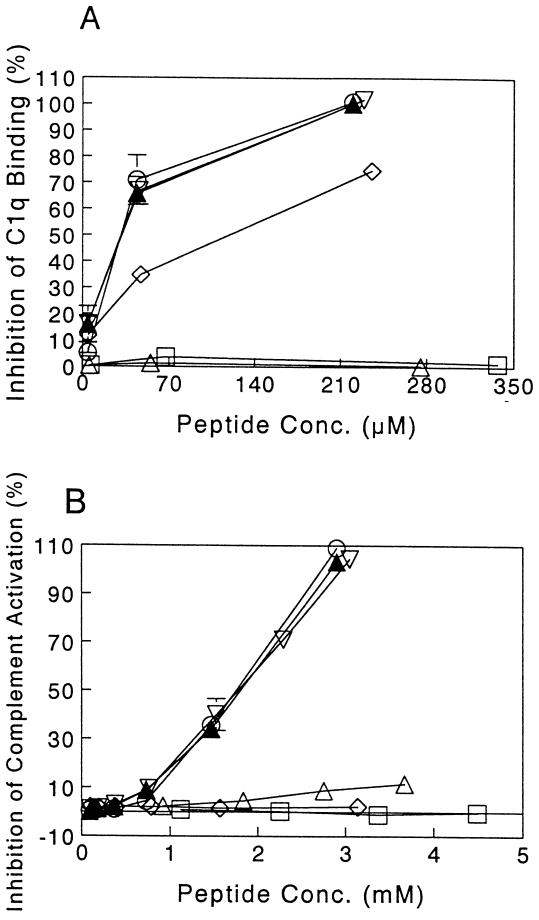

Fig. 3. C1qA peptides inhibit C1q binding to and complement activation by CL20 liposomes. C1q binding to CL20 liposomes in the presence of peptides at pH 7.2 was measured using the C1q equilibrium binding assay (panel A). The reaction mixture consisted of 0.5 mM total lipid, 9 nM C1q, and peptides at different concentrations. The amount of complement consumed (activated) by CL20 liposomes (5 mM) in human serum (diluted 1:4) after an initial incubation of liposomes and peptides was measured using a modified hemolytic assay (panel B). The percent inhibition of C1q binding (panel A) or complement activation (panel B) is shown for the authentic $C1qA_{(14–26)}$ (▲), $C1qA_{(scrambled)}$ (○), $C1qA_{(P–A)}$ (▽), $C1qA_{(0+)}$ (□), $C1qA_{(2+)}$ (△), and the unrelated Fgn (◇) peptides. Data points show the mean of three experiments. Error bars represent one standard deviation. Where no error bars are shown, one S.D. was smaller than the size of the symbol used for the mean.

than two cationic residues were required to interact with anionic liposomes. The role of the amino terminal charge in the peptides was not directly assessed; however, since $C1qA_{(0+)}$ had no effect on binding, it is unlikely that the amino terminal charge of the peptides plays a significant role. One peptide, $C1qA_{(scrambled)}$, was also prepared with a blocked carboxy terminal. Its ability to block C1q binding was unaffected by this modification (data not shown). Surprisingly, the unrelated Fgn peptide at 240 μM inhibited 70% of C1q binding to CL20 liposomes.

3.4. Inhibition of liposome complement activation by C1qA peptides

A modified hemolytic assay was used to measure the ability of C1qA and control peptides to inhibit complement activation by liposomes in human serum (Fig. 3B). $C1qA_{(14–26)}$ completely blocked complement activation by CL20 liposomes at a concentration of 3 mM in this system and produced 40% inhibition at approximately 1.7 mM. Control C1qA peptides (scrambled and P-A peptides) inhibited complement activation to the same extent as the authentic peptide verifying that the interaction lacks sequence and conformational specificity. Charge control peptides, $C1qA_{(0+)}$ and $C1qA_{(2+)}$, failed to inhibit complement activation by CL20 liposomes confirming that more than two positively charged residues are required for effective interaction of C1q with anionic liposomes. The unrelated Fgn peptide failed to inhibit complement activation. Peptides incubated with EA cells alone did not cause cell lysis and peptides incubated with NHS in the absence of liposomes did not activate complement.

3.5. Direct interaction of cationic peptides with anionic liposomes

The direct interaction between anionic liposomes and C1q peptides was assessed by particle electrophoresis at pH 7 under interaction-promoting low ionic strength conditions (Fig. 4). Authentic $C1qA_{(14–26)}$, $C1qA_{(scrambled)}$, and $C1qA_{(P–A)}$ peptides all interacted strongly with CL20 MLVs. In contrast, no interaction was detected with the $C1qA_{(0+)}$ peptide. While some peptide–MLV binding was observed at the highest concentration of the $C1qA_{(2+)}$ peptide (900 μg/ml), it was less than the Fgn peptide. The magnitude of these direct interactions between C1qA peptides and CL20 MLVs mirrored the inhibitory capacities of the peptides.

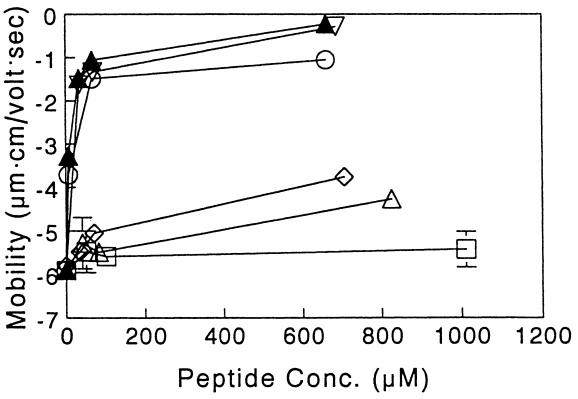

Fig. 4. Interaction of C1qA peptides with CL20 liposomes. Particle electrophoresis was used to monitor the direct interaction of C1qA and control peptides with CL20 MLVs at pH 7 and 20 mM NaCl. Peptides at 9, 45, 90, or 900 μg/ml were incubated with MLVs (0.36 mM total lipid in reaction) for 40 min at room temperature. Liposome mobilities in the presence of increasing amounts of $C1qA_{14–26}$ (▲), $C1qA_{(scrambled)}$ (○), $C1qA_{(P–A)}$ (▽), $C1qA_{(0+)}$ (□), $C1qA_{(2+)}$ (△), and unrelated Fgn (◇) peptides are presented. Mobility measurements were made on ten vesicles in the mixture each time. The complete experiment was carried out three times. Error bars represent one standard deviation. Where no error bars are shown, one S.D. was smaller than the size of the symbol used for the mean.

To further characterize peptide–CL20 MLV interactions, the effect of ionic strength was investigated using particle electrophoresis. Mobility measurements were made on ten vesicles in the mixture each time in 3 complete experiments. CL20 MLVs alone had mobilities of -5.94 ± 0.18 and -3.48 ± 0.17 (μm•cm/V•s ± S.D.) in 20 mM and 100 mM NaCl, respectively. When peptides (90 μg/ml) were incubated with CL20 MLVs, a mobility shift towards zero indicated that peptide binding to liposomes had occurred. $C1qA_{(14–26)}$ and the sequence and conformation control peptides, $C1qA_{(scrambled)}$ and $C1qA_{(P–A)}$, exhibited a high level of liposome binding with more binding at lower ionic strength. Liposome mobilities in 20 mM NaCl were -1.06 ± 0.11, -1.49 ± 0.08, and -1.33 ± 0.03 (μm•cm/V•s) for $C1qA_{(14–26)}$, $C1qA_{(scrambled)}$ and $C1qA_{(P–A)}$ respectively. In 100 mM NaCl, mobilities

were -1.74 ± 0.11, -1.95 ± 0.06, and -1.68 ± 0.09 (μm•cm/V•s) for these peptides. While $C1qA_{(0+)}$ and $C1qA_{(2+)}$ peptides did not bind to CL20 MLVs, the Fgn peptide exhibited a slight binding capacity but only at 20 mM NaCl (liposome mobility: -5.05 ± 0.13 (μm•cm/V•s)).

At pH 7, liposomes aggregated under conditions where C1qA peptide binding was enhanced: at low ionic strength and when highly charged peptides were used. Even at 20 mM NaCl, when C1q(0+) or Fgn peptides were used, no aggregation was observed. For the highly charged peptides, the authentic $C1qA_{(14-26)}$, $C1qA_{(scramble)}$, and $C1qA_{(P-A)}$, liposome aggregation was proportional to the amount of peptide added.

The techniques employed in these studies necessitated using liposomes of different size. Our earlier studies of the effects of liposome composition on complement activation were carried out using unilamellar 100-nm vesicles [24,27]; however, this size of liposomes could not be used for either the equilibrium binding studies or the particle electrophoresis studies. It is possible that the findings are somewhat influenced by liposome size. We have previously demonstrated that for a constant amount of phospholipid, larger vesicles ($\sim$260 nm) are more effective complement activators than smaller vesicles (70 nm) [27]. Thus, we may have underestimated the amount of C1q binding in functional complement assays relative to that measured in equilibrium binding studies.

4. Discussion

Anionic liposomes can activate the classical pathway of complement in either an antibody-dependent or in an antibody-independent manner [13,24]. Since complement activation may lead to the rapid loss of liposomes in the blood, we wished to gain a better understanding of the factors involved in the direct binding of C1q to anionic liposomes.

Under physiologic pH and ionic strength, a small but measurable amount of C1q bound to anionic liposomes. The saturation binding value for CL20 liposomes was 2.2×10^{-6} μmol C1q bound per μmol of total lipid or 3 C1q molecules for every two liposomes. This low level of binding of C1q was somewhat surprising since these liposomes have been previously shown to strongly activate complement in a full complement activation assay [13]. Others have made related comparisons, however. Kovacsovics et al. [28] found that C1 activation was more readily detected than C1q binding. In general, the binding of small amounts of C1q to activator surfaces still results in an irreversible activation event. Also, the cascade nature of complement activation allows for a greater complement response than the level of C1q binding might appear to warrant.

While C1q binding was small at physiologic pH and ionic strength, the binding capacity was enhanced by increasing the surface charge density of the anionic liposomes. As the proportion of anionic phospholipid in the liposomes increased, increases in C1q binding at saturation were measured (Fig. 1, Table 1). This relationship between surface charge density and C1q binding parallels the effect of anionic phospholipid concentration on overall complement activation reported in several studies [1,24,27]. Our results confirm and expand upon observations by Kovacsovics et al. [28] of the charge density dependence of C1q binding and activation of C1 by cardiolipin-containing liposomes.

The fact that liposome surface charge density strongly affects the level of C1q binding to liposomes indicates that electrostatic attraction is necessary for C1q to bind to liposomes. Negative cooperativity, i.e., a decrease in apparent association constant as binding proceeds, would be expected since the surface charge on the liposome decreases as ligand binding proceeds. However, this was not observed as the data sets all fit the simple binding equation satisfactorily with a single association constant. Moreover, while the saturation level of C1q binding increased with increasing CL, the association constant was independent of the concentration of charged lipid in the liposomes (Table 1). This is opposite to what is expected for a purely electrostatic model in which the electrostatic attraction can strongly enhance the apparent association constant [29]. This may suggest that the physical binding of C1q to the liposome surface relies on hydrophobic interactions as well as electrostatic ones and that the binding reaction is not as simple as implied by the equation utilized to fit the individual data sets. De Kroon [30] has

suggested that both electrostatic and hydrophobic forces are involved in the binding of positively charged peptides to CL-containing vesicles.

The effects of ionic strength and pH on C1q–liposome interactions were assessed using particle electrophoresis. The electrophoretic mobility of a particle is measured as the velocity of a vesicle in an applied electric field and is directly related to the surface charge density of the liposome [31]. When C1q was added, the binding of C1q to liposomes decreased the magnitude of the liposome electrophoretic mobility by masking liposome surface charge and replacement by exposed charges on the liposome-bound proteins. Consequently, the decrease in liposome electrophoretic mobility is directly related to the amount of C1q bound. It is important to note that due to the experimental design for particle electrophoresis studies, C1q–liposome interactions may be underestimated by these measurements. To conserve purified C1q, the reaction mixture volume was kept at 780 μl, then diluted with buffer to fill the 2.8-ml sample chamber. Dilution immediately prior to making the electrophoretic mobility measurements may have displaced some C1q from the liposomes. This dilution effect probably explains why C1q binding to MLVs at physiologic pH and the ionic strength seen in the equilibrium binding assay was not detected by particle electrophoresis.

A much greater relative change in the electrophoretic mobility of the liposomes was observed in the presence of C1q as the ionic strength was lowered from physiologic 145 mM to 20 mM, reflecting enhanced C1q binding to CL20 MLVs as the ionic strength was decreased. The enhancement of C1q binding by lowering the ionic strength was anticipated since many studies of C1q binding to other substances utilized low ionic strength conditions [32–35]. Even C1q binding to specific receptors on cells and platelets is ionic strength dependent [36].

C1q binding to anionic liposomes was also highly dependent upon the pH of the environment with more C1q binding at pH 5 than at pH 7. The enhancement of protein binding to anionic liposomes by decreasing the pH has been reported for other proteins [30,37]. With respect to electrostatics, C1q recruitment to the liposome surface by attraction between membrane- and protein-associated charges would be strongest at low pH.

The aggregation state of C1q may also contribute to the different binding levels at decreased ionic strength and pH. Aggregated C1q would be expected to bind more strongly due to multivalent interactions with liposomes. C1q aggregation occurs when the ionic strength is reduced below 100 mM NaCl [38] and is implied here by the observation that liposomes aggregated under conditions where higher levels of C1q binding occurred (low pH and low ionic strength). Liposome aggregation may be mediated by C1q aggregation although the reduced liposome surface potential as a result of C1q binding will also contribute.

We examined a candidate region of C1q which could mediate the interaction of C1q with anionic liposomes: residues 14–26 within the collagen-like region of the C1qA chain. As described for the antibody-independent complement activators, SAP, CRP, DNA and β-amyloid, $C1qA_{(14–26)}$ peptide was capable of completely inhibiting C1q binding and complement activation by anionic liposomes [17–20]. Inhibition of C1q binding to anionic liposomes required the same relative amount of $C1qA_{(14–26)}$ peptide as that required for inhibition of C1q binding to CRP or to DNA. However, this amount is very large; a molar ratio of 1.8×10^4:1 (peptide:C1q) was needed to inhibit C1q binding by 90%. Similar ratios have been reported by others [19]. High concentrations of peptides may be required to outcompete C1q since C1q may contain six or more binding sites.

In marked contrast to studies on other direct complement activators [17–19], the capacity of the $C1qA_{(14–26)}$ peptide to inhibit C1q binding and complement activation by anionic liposomes lacked any sequence or conformational specificity. $C1qA_{(scrambled)}$ and $C1qA_{(P–A)}$ peptides exhibited the same inhibitory abilities as the authentic $C1qA_{(14–26)}$ peptide. In addition, all three peptides interacted directly with anionic liposomes to virtually the same extent. Thus, C1q binding and complement activation on a phospholipid membrane differs from binding and activation by protein, DNA or fiber aggregates.

The lack of sequence and conformation specificity in the interaction between C1q and anionic liposomes indicates that the charge component was of prime importance. Only peptides with a large net

positive charge (+5) were capable of interacting with anionic liposomes and inhibiting C1q binding and complement activation. Peptides with two cationic charges bound slightly at very low ionic strength, not at all in 100 mM NaCl and had no inhibitory effects. For the neutral peptide ($C1qA_{(0+)}$), no binding to liposomes and no inhibitory capacity was detected. In addition, the inverse relationship between ionic strength and C1qA peptide–liposome binding mirrored the ionic strength dependence shown for C1q–liposome binding (Fig. 2) and for C1q–DNA interactions [19]. The unmodified peptide sequence is derived from a collagen-like region in the C1q A-chain. If one aligns the peptide sequence in a collagen helix ($n=3$ residues per turn), the peptide shows the classical collagen alignment of all glycine residues to form an apolar side to the helix and proline residues aligned on a second side. The third side of the helix contains three of the five cationic residues. Although this alignment, especially of the glycine residues, is thought to facilitate collagen's tertiary and quaternary structure, it is unlikely to be the major factor in peptide inhibition of C1q binding. The alignment of the scrambled sequence places cationic residues on all sides of a collagen helix, yet the scrambled peptide effectively inhibited C1q binding to anionic liposomes.

Since C1qA peptides of sufficient charge (possibly more than two and certainly five cationic residues) were capable of successfully inhibiting C1q-mediated complement activation by anionic liposomes regardless of the primary sequence or conformation of the peptide, other regions of the protein with a similar expanse of residues may also be involved. Another cationic region of the C1qA chain, residues 76–92 at the hinge region of the structure, is of secondary importance in mediating the binding of C1q to DNA [19], to CRP trimers [18] and to SAP trimers [17]. Residues 76–92 include four cationic amino acids and one anionic residue, giving a net charge at physiologic pH of +3. This region is therefore a candidate site on C1q for an electrostatic interaction with anionic liposomes.

Partial inhibition of C1q binding to anionic liposomes by the Fgn peptide (having an overall charge of approximately +1.2 at pH 7.2) was unexpected. The Fgn peptide neither bound anionic MLVs at 100 mM NaCl nor inhibited complement activation indicating that the mechanism by which the Fgn peptide blocked C1q–liposome binding was different from that of C1qA peptides. Fibrinogen binds directly to C1q through at least two sites: one each on the collagenous and the globular regions [32]. The Fgn peptide may bind to C1q near the liposome binding site and thus interfere with C1q–liposome binding. The failure of the Fgn peptide to block complement activation by anionic liposomes may be due to alteration of the C1q–Fgn peptide interaction by other serum proteins.

These experiments characterizing the binding of C1q to anionic liposomes collectively have shown that electrostatics play a dominant role in the interaction between C1q and liposomes. C1q–liposome binding depended on liposome surface charge and on the pH and ionic strength of the environment. In addition, competitive C1q binding and complement activation experiments with peptides at physiologic pH and ionic strength as well as direct measurements of peptide–liposome interactions identified the electrostatic component of the C1q–liposome interaction as being important. However, other chemical forces including hydrophobic interactions may also contribute to the actual binding energy.

Acknowledgements

We would like to thank Dr. Stuart McLaughlin, SUNY at Stony Brook, for useful discussions and Dr. Andrea Tenner, U.C., Irvine, for advice regarding the C1q purification and radiolabeling. This work was supported by grants in aid from the Medical Research Council of Canada (MT-10933 to D.V.D. and MT-5759 to D.E.B.).

References

[1] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, Biochim. Biophys. Acta 1113 (1992) 171–199.
[2] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood

proteins with large unilamellar liposomes in vivo, J. Biol. Chem. 267 (1992) 18759–18765.
[3] S.M. Moghimi, H.M. Patel, Opsonophagocytosis of liposomes by peritoneal macrophages and bone marrow reticuloendothelial cells, Biochim. Biophys. Acta 1135 (1992) 269–274.
[4] D.V. Devine, A.J. Bradley, The complement system in liposome clearance: can complement deposition be inhibited?, Adv. Drug Deliv. Rev. 32 (1998) 19–29.
[5] K. Funato, R. Yoda, H. Kiwada, Contribution of complement system on destabilization of liposomes composed of hydrogenated egg phosphatidylcholine in rat plasma, Biochim. Biophys. Acta 1103 (1992) 198–204.
[6] T.R. Hesketh, R.R. Dourmashkin, S.N. Payne, J.H. Humphrey, P.J. Lachmann, Lesions due to complement in lipid membranes, Nature 233 (1971) 620–623.
[7] H. Matsuo, K. Funato, H. Harashima, H. Kiwada, The complement- but not mannose receptor-mediated phagocytosis is involved in the hepatic uptake of cetylmannoside-modified liposomes in situ, J. Drug Target. 2 (1994) 141–146.
[8] J.F. Scieszka, L.L. Maggiora, S.D. Wright, M.J. Cho, Role of complements C3 and C5 in the phagocytosis of liposomes by human neutrophils, Pharm. Res. 8 (1991) 65–69.
[9] H. Harashima, K. Sakata, K. Funato, H. Kiwada, Enhanced hepatic uptake of liposomes through complement activation depending on the size of the liposomes, Pharm. Res. 1 (1994) 402–406.
[10] D. Liu, F. Liu, Y.K. Song, Recognition and clearance of liposomes containing PS are mediated by serum opsonin, Biochim. Biophys. Acta 1235 (1995) 140–146.
[11] F. Roerdink, N.M. Wassef, E.C. Richardson, C.R. Alving, Effects of negatively charged lipids on phagocytosis of liposomes by complement, Biochim. Biophys. Acta 734 (1983) 33–39.
[12] N.M. Wassef, C.R. Alving, Complement-dependent phagocytosis of liposomes, Chem. Phys. Lipids 64 (1993) 239–248.
[13] J. Marjan, Z. Xie, D.V. Devine, Liposome-induced activation of the classical complement pathway does not require immunoglobulin, Biochim. Biophys. Acta 1192 (1994) 35–44.
[14] K.B.M. Reid, R.R. Porter, Subunit composition and structure of subcomponent C1q of the first component of human complement, Biochem. J. 155 (1976) 19–23.
[15] K.B.M. Reid, J. Gagnon, J. Frampton, Completion of the amino acid sequences of the A and B chains of subcomponent C1q of the first component of human complement, Biochem. J. 203 (1982) 559–569.
[16] C.J. Strang, R.C. Siegel, M.L. Phillips, P.H. Poon, V.N. Schumaker, Ultrastructure of the first component of human complement: electron microscopy of the crosslinked complex, Proc. Natl. Acad. Sci. USA 79 (1982) 586–590.
[17] S. Ying, A.T. Gewurz, H. Jiang, H. Gewurz, Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14–26 and 76–92 of the A chain collagen-like region of C1q, J. Immunol. 150 (1993) 169–176.
[18] H. Jiang, F.A. Robey, H. Gewurz, Localization of sites through which C-reactive protein binds and activates complement to residues 14–26 and 76–92 of the human C1q A chain, J. Exp. Med. 176 (1992) 1373–1379.
[19] H. Jiang, B. Cooper, F.A. Robey, H. Gewurz, DNA binds and activates complement via residues 14–26 of the human C1q A chain, J. Biol. Chem. 267 (1992) 25597–25601.
[20] H. Jiang, D. Burdick, C.G. Glabe, C.W. Cotman, A.J. Tenner, β-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain, J. Immunol. 152 (1994) 5050–5059.
[21] A.J. Tenner, P.H. Lesavre, N.R. Cooper, Purification and radiolabeling of human C1q, J. Immunol. 127 (1981) 648–653.
[22] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.
[23] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1924) 375–400.
[24] A. Chonn, P.R. Cullis, D.V. Devine, The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes, J. Immunol. 146 (1991) 4234–4241.
[25] J. Janzen, X. Song, D.E. Brooks, Interfacial thickness of liposomes containing poly(ethylene glycol)-cholesterol from electrophoresis, Biophys. J. 70 (1996) 313–320.
[26] G.V.F. Seaman, Electrokinetic behaviour of red cells, in: D.M. Surgenor, (Ed.), The Red Blood Cell, Academic Press, New York, 1975, pp. 1135–1229.
[27] D.V. Devine, K. Wong, K. Serrano, A. Chonn, P.R. Cullis, Liposome–complement interactions in rat serum: implications for liposome survival studies, Biochim. Biophys. Acta 1191 (1994) 43–51.
[28] T. Kovacsovics, J. Tschopp, A. Kress, H. Isliker, Antibody-independent activation of C1, the first component of complement, by cardiolipin, J. Immunol. 135 (1985) 2695–2700.
[29] D. Shaw, Charged interfaces, in: D. Shaw (Ed.), Introduction to Colloid and Surface Chemistry, Butterworth, London, 1970, pp. 133–166.
[30] A.I.P.M. De Kroon, M.W. Soekarjo, J. De Gier, B. De Kruijff, The role of charge and hydrophobicity in peptide–lipid interaction, Biochemistry 29 (1990) 8229–8240.
[31] M. Mosier, S. McLaughlin, Binding of basic peptides to acidic lipids in membranes: effects of inserting alanine(s) between the basic residues, Biochemistry 31 (1992) 1767–1773.
[32] R.A. Entwistle, L.T. Furcht, C1q component of complement binds fibrinogen and fibrin, Biochemistry 27 (1988) 507–512.
[33] J. Sorvillo, I. Gigli, E. Pearlstein, Fibronectin binding to complement subcomponent C1q: localization of their respective binding sites, Biochem. J. 226 (1985) 207–215.

[34] E.J. Menzel, J. Smolen, K. Reid, Interaction of collagen with C1q via its collagen-like portion, Biochim. Biophys. Acta 670 (1981) 265–273.
[35] A. Zohair, S. Chesne, R.H. Wade, M.G. Colomb, Interaction between complement subcomponent C1q and bacterial lipopolysaccharides, Biochem. J. 257 (1989) 865–873.
[36] R.D. Schreiber, The chemistry and biology of complement receptors, Springer Semin. Immunopathol. 7 (1984) 221–249.
[37] J.J. Bergers, M.H. Vingerhoeds, L. van Bloois, J.N. Herron, L.H.M. Janssen, M.J.E. Fischer, D.J.A. Crommelin, The role of protein charge in protein–lipid interactions. pH-dependent changes of the electrophoretic mobility of liposomes through adsorption of water-soluble, globular proteins, Biochemistry 32 (1993) 4641–4649.
[38] R.J. Ziccardi, N.R. Cooper, Active disassembly of the first component of complement, C1, by C1-inactivator, J. Immunol. 123 (1979) 788–792.