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RESEARCH ARTICLE

Complement activation by phospholipids: the interplay of factor H and C1q

Lee Aun Tan[1,4], Bingbin Yu[1,5], Francis CJ Sim[2], Uday Kishore[3], Robert B Sim[1,2] ✉

[1] MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Rd, Oxford OX1 3QU, UK
[2] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK
[3] Centre for Infection, Immunity and Disease Mechanisms, Biosciences, School of Health Sciences and Social Care, Brunel University, Uxbridge UB8 3PH West London, UK
[4] Present address Department of Immunology, Faculty of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
[5] Present address School of Life Sciences and Engineering, Biology Building, Tsinghua University, Beijing 100084, China
✉ Correspondence: bob.sim@pharm.ox.ac.uk
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ABSTRACT

Complement proteins in blood recognize charged particles. The anionic phospholipid (aPL) cardiolipin binds both complement proteins C1q and factor H. C1q is an activator of the complement classical pathway, while factor H is an inhibitor of the alternative pathway. To examine opposing effects of C1q and factor H on complement activation by aPL, we surveyed C1q and factor H binding, and complement activation by aPL, either coated on microtitre plates or in liposomes. Both C1q and factor H bound to all aPL tested, and competed directly with each other for binding. All the aPL activated the complement classical pathway, but negligibly the alternative pathway, consistent with accepted roles of C1q and factor H. However, in this system, factor H, by competing directly with C1q for binding to aPL, acts as a direct regulator of the complement classical pathway. This regulatory mechanism is distinct from its action on the alternative pathway. Regulation of classical pathway activation by factor H was confirmed by measuring C4 activation by aPL in human sera in which the C1q:factor H molar ratio was adjusted over a wide range. Thus factor H, which is regarded as a down-regulator only of the alternative pathway, has a distinct role in downregulating activation of the classical complement pathway by aPL. A factor H homologue, β2-glycoprotein-1, also strongly inhibits C1q binding to cardiolipin. Recombinant globular domains of C1q A, B and C chains bound aPL similarly to native C1q, confirming that C1q binds aPL via its globular heads.

KEYWORDS complement, regulation, classical pathway, C1q, factor H, anionic phospholipid

INTRODUCTION

The complement system is a major contributor to humoral immunity, mediating many activities that contribute to host defense and opsonization for phagocytosis. More than 30 proteins, both in serum and on cell surfaces are part of this system (Kang et al., 2009). Complement recognizes a wide range of targets including microorganisms, altered host cells and synthetic particles. Targets are recognized by proteins of the three activation pathways, C1q from the classical pathway, Mannan binding lectin (MBL) and ficolins from the lectin pathway and C3, factors H and B and properdin from the alternative pathway. Complement activation by the classical pathway occurs when the first component of complement, C1, binds, mainly via charge interactions (Roumenina et al., 2007) to immune complexes (containing IgG or IgM) or directly to a non-immunoglobulin target surface. The antibody-independent activation of C1 has been described for a wide variety of organisms and substances (Sim and Malhotra, 1994) including DNA (Agnello et al., 1969), Gram-negative bacteria (Clas et al., 1989), retroviruses (Cooper et al., 1976), amyloids and prions (Sim et al., 2007), anionic phospholipids (Marjan et al., 1994) and synthetic materials (Rybak-Smith et al., 2010). C1 is a complex of three glycoproteins, C1q, C1r and C1s. C1r and C1s are proteases which are activated when C1q binds to a target. Complement activation by the alternative pathway occurs when C3, factor B and properdin interact with particulate matter such as bacterial and fungal cell wall

constituents, IgG immune complexes, or a wide variety of other compounds and surfaces (Sim and Malhotra, 1994). Conversely, complement factor H binding to surfaces and/or to C3b on surfaces inhibits activation of the alternative pathway (Carreno et al., 1989). MBL and ficolins, serum proteins with structural similarity to C1q, bind mainly via nonionic interactions to glycans and other targets, leading to activation of the lectin pathway via the MBL-associated serine protease 2 (MASP-2) which is homologous to C1r and C1s (Wallis et al., 2010).

Anionic phospholipids are known complement classical pathway activators (Chonn et al., 1991; Marjan et al., 1994; Tan and Sim, 2001). Cardiolipin (CL), a negatively charged phospholipid found abundantly in mitochondrial membranes binds C1q and activates the classical pathway in an antibody-independent manner (Kovacsovics et al., 1985). C1q also binds to phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) (Marjan et al., 1994; Tan et al., 2002; Païdassi et al., 2008). However, in separate work, the complement downregulator, factor H, has also been shown to bind strongly to cardiolipin (Kertesz et al., 1995), but its binding to other anionic phospholipids has not been investigated. Although C1q and factor H are not similar in structure, both bind mainly by charge interactions to a wide range of macromolecules.

C1q is a 460 kDa glycoprotein composed of 18 polypeptide chains (6A–C) and is present in human serum at a concentration of about 115 mg/L (Reid and Porter, 1976; Dillon et al., 2009). The A, B and C chains combine to form six equivalent subunits each consisting of a collagen-like “stalk” at the N terminus of the molecule and a carboxyl-terminal heterotrimeric globular (gC1q) domain. The six subunits associate to form a structure resembling a bunch of six tulips when viewed under the electron microscope. The C1r and C1s subcomponents of C1 bind the collagen region of C1q. Binding of C1q to ligands brings about a conformational change in the collagen region that activates C1r and C1s (Dodds et al., 1978). C1q binds to IgG and IgM-containing immune complexes via its gC1q domains. Non-antibody ligands also bind generally but perhaps not exclusively via the gC1q domain (Tacnet-Delorme et al., 2001).

Factor H, a single polypeptide chain protein of 155 kDa, is composed of 20 complement control protein (CCP) modules (Ripoche et al., 1988a). Its concentration in human sera is reported to vary quite widely between individuals (150–750 mg/L: Charlesworth et al., 1979; Edey et al., 2009; Ingram et al., 2010). It prevents the assembly of and dissociates the alternative complement pathway C3 convertase, C3bBb, and acts as a cofactor for factor I-mediated cleavage of C3b (Sim et al., 1993). The discrimination between activators and non-activators of the alternative pathway is determined by binding of factor H to surfaces and to surface-associated C3b (Carreno et al., 1989). The interaction of factor H with surface charge clusters and C3b is of major importance in the activation and regulation of complement.

C1q represents a forward acting component as it serves to activate complement whereas factor H serves to down-regulate complement activation. Here we examined the interactions of C1q and factor H with various phospholipids and their ability to activate the whole complement system. Using solid phase phospholipids (immobilised on microtiter wells) and multilamellar phospholipid liposomes, we found that both C1q and factor H bound to anionic phospholipids. The binding of immunoglobulins to phospholipid-coated wells had only small effects on the subsequent binding of C1q to phospholipids. The significance of C1q binding to phospholipids was demonstrated by the activation (consumption) of complement. Factor H was able to compete strongly with C1q for binding to CL, thus acting as a regulator of the activation of the classical pathway. Using purified recombinant globular head regions of the A, B and C chains of C1q, we found that each of the globular head chains contributed to the binding of C1q to phospholipids.

RESULTS

Interaction of ^{125}I-C1q and ^{125}I-factor H with phospholipid-coated wells

Purified human ^{125}I-C1q or ^{125}I-factor H was incubated in microtiter plate wells coated with one of five anionic phospholipids (PA, CL, PS, PG, PI) or one of two neutral phospholipids (PE and PC). C1q bound to anionic PA, CL, PS and neutral PE with much lower binding to PG and PI (Fig. 1)

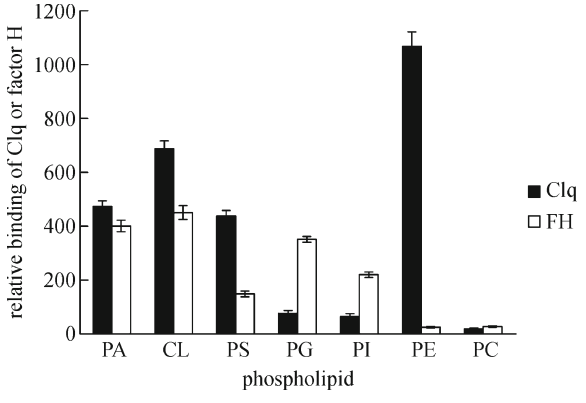

Figure 1. Binding of ^{125}I-C1q and ^{125}I-factor H (FH) to solid-phase bound phospholipids. A fixed amount of ^{125}I-C1q (10^4 cpm/well; black bars) or ^{125}I-factor H (10^4 cpm/well; white bars) was incubated with various phospholipid-coated wells (5 μg phospholipid/well) for 1 h at RT in VB^{2+}. The wells were washed, and the amount of bound C1q or factor H was measured (in cpm/well). Binding to control wells (containing no phospholipids but blocked with ovalbumin) was negligible. Error bars represent standard deviation for five experiments.

and very low binding to PC. Factor H bound to all the anionic phospholipids tested (Fig. 1). Strong binding was observed to PA, CL, PG and PI with lesser binding to PS, and very low binding to neutral PE and PC. The quantity of C1q bound to CL was comparable to its binding to wells coated with 5 μg/well of rabbit IgG Fc fragment (not shown). To ensure that the differences detected in ^{125}I-C1q or ^{125}I-factor H binding (Fig. 1) were not caused by differences in the amount of phospholipids adsorbed to the microtiter wells, we quantified (Stewart, 1980) the solid-phase phospholipids by eluting with methanol. The amount bound varied from a minimum of 250 ng/well (PA) to a maximum of 290 ng/well (PG and PC). This variation is not sufficient to explain the differences in ^{125}I-C1q and ^{125}I-factor H binding.

Interaction of ^{125}I-C1q and ^{125}I-factor H with phospholipid liposomes

We examined if C1q or factor H bound to phospholipid in liposomes. Both C1q and factor H (Fig. 2) bound strongly to CL liposomes. The binding of C1q to PA, PS, PG and PI liposomes was lower than to CL but still above the background level seen with the control (PC liposomes). PE liposomes bind slightly more factor H than the control, but C1q binding to PE liposomes was not above the control level.

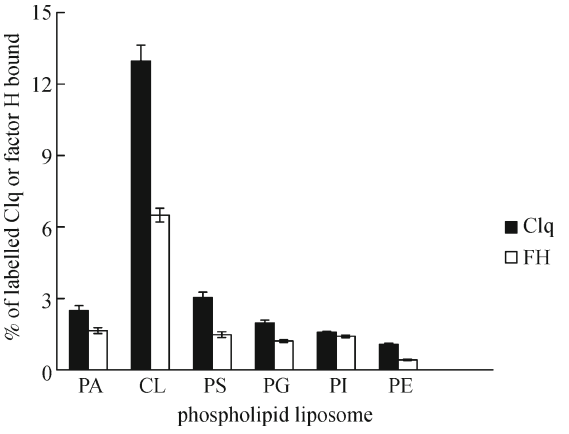

Figure 2. Binding of ^{125}I-C1q and ^{125}I-factor H to phospholipid liposomes. A fixed amount of ^{125}I-C1q (90,000 cpm/reaction; black bars) or ^{125}I-factor H (10^5 cpm/reaction; white bars) in VB^{2+} was incubated with various multilamellar phospholipid liposomes (10 μg/reaction) for 1 h at RT. The liposomes were washed and the amount of C1q or factor H bound was quantified. Liposomes consisting of 55 molar percent DPPC and 45 molar percent CHOL were used as controls. Binding percentages were obtained after deducting control values. Error bars represent standard deviation for five experiments.

Binding of proteins from whole human serum to CL-coated wells

To identify other proteins in human serum which can bind to CL, human serum was loaded onto CL-coated wells on a 24 well plate and the bound proteins were analyzed by SDS-PAGE. As shown in Fig. 3A, there were two major components in the CL binding fractions together with some minor components. The major ones are β_2-1 and factor H, as shown by their respective co-migration with a β_2-1 and factor H standard under nonreducing (Fig. 3A) and reducing (not shown) conditions. Their identities were also confirmed by N-terminal amino acid sequencing (not shown). C1q was found from its co-migration with a C1q standard (Fig. 3A), and in these conditions, it bound in lower quantity than factor H or β_2-1.

Inhibition of C1q binding to CL by factor H and β_2-1

Purified β_2-1 competes with purified factor H for binding to CL (Kertesz et al., 1995) and factor H and β_2-1 are two of the major serum proteins binding to CL. We investigated whether factor H and β_2-1 compete with C1q for binding to CL. As shown in Fig. 3B, both factor H and β_2-1 potently inhibit the binding of ^{125}I-C1q to CL-coated wells. Factor H also competes with ^{125}I-C1q for binding to CL liposomes. This is consistent with the comparable binding of C1q and factor H to CL at saturation (Table 1). These results (Fig. 3A and 3B) show that factor H and β_2-1 compete with C1q in serum for binding to CL. A maximum of only ~70% inhibition was observed at a 3:1 molar ratio of factor H or β_2-1 to C1q. This ratio is however lower than the ratio in human sera, which for factor H:C1q is in the range about 6:1 up to about 30:1.

A previous report indicated that rheumatoid arthritic protein (RHP), which was characterized as being antigenically related to factor H, was able to bind to the gC1q domain of C1q (Holme et al., 1992). In another report (Easterbrook-Smith et al., 1992), factor H was suggested to bind directly to C1q. Since factor H was able to compete with C1q for binding to CL (Fig. 3B), we sought to clarify whether the effect of factor H in inhibiting C1q binding was due to the interaction of factor H with CL, or factor H interaction with C1q. When ^{125}I-C1q was incubated with factor H-coated wells, there was no detectable binding of C1q (not shown). Furthermore, on C1q-coated wells, factor H did not show any binding (not shown). Thus, factor H appeared to inhibit C1q binding to CL through direct competition for binding sites on CL.

Inhibition of ^{125}I-C1q binding to CL-coated wells by human serum

NHS and C1q-depleted human serum were found to inhibit the binding of ^{125}I-C1q to CL in a concentration-dependent manner. NHS inhibited binding of ^{125}I-C1q more strongly than did C1q-depleted serum (Fig. 4). This difference could be attributed to added competition in NHS by the C1 complex ($C1q\text{-}C1r_2\text{-}C1s_2$) in addition to the inhibition by factor H and β_2-1, which are present in both NHS and C1q-depleted

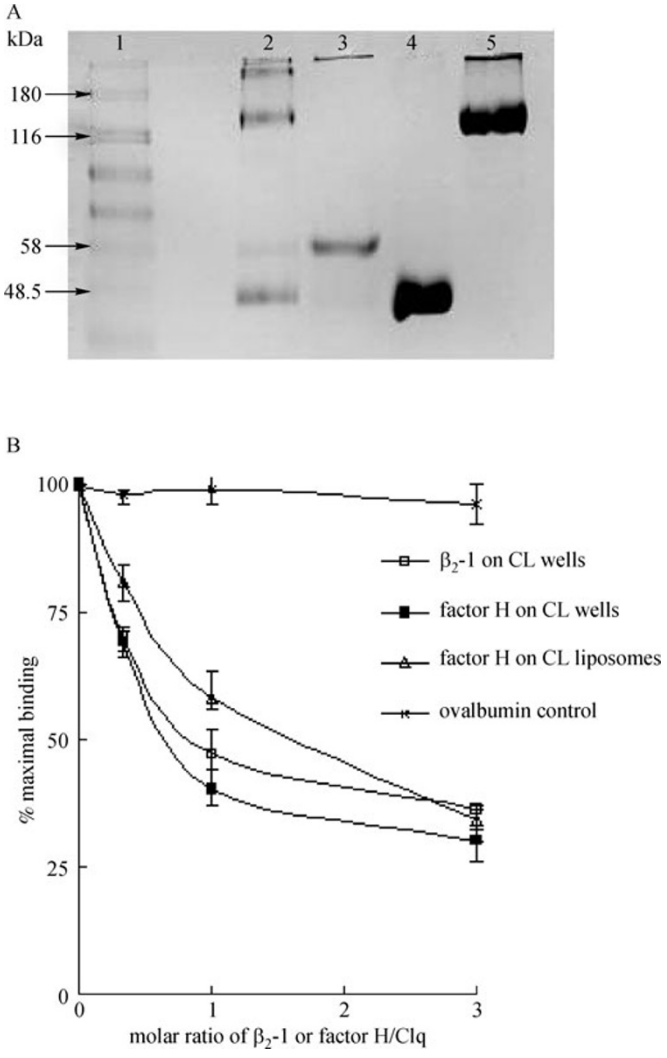

Figure 3. Analysis of serum proteins bound to CL-coated wells. (A) Human serum was added to the CL-coated wells and proteins that remained bound after thorough washing were analyzed by SDS-PAGE (9.5% *w/v* acrylamide gel under nonreducing conditions). Lane 1, molecular mass markers; lane 2, eluate from CL coated wells after incubation of pooled human serum for 2 h at RT in CL-coated wells; lane 3, human C1q standard; lane 4, human β_2-1; lane 5, human factor H. The C1q standard shows the AB dimer at ~55 kDa. The CC dimer is partially aggregated under these conditions (Reid and Porter, 1976) but shows weakly at ~44 kDa. (B) Inhibition of ^{125}I-C1q binding to CL by purified serum proteins. A fixed amount of ^{125}I-C1q in VB^{2+} (10^5 cpm, 3 μg of protein) in VB^{2+} was incubated with various molar ratios of unlabelled purified factor H or β_2-1 glycoprotein on CL-coated wells or with unlabelled factor H and CL liposomes. The CL-coated wells or CL liposome pellet were washed and the amount of ^{125}I-C1q bound was determined and expressed as a percentage of the C1q bound in the absence of competing protein. Ovalbumin was used as a control protein. Error bars represent standard deviation for three experiments.

serum. These results further corroborated the observations from Fig. 3A where factor H and β_2-1 were found to be the two major CL binding species with much smaller quantities of C1q binding. Factor H and β_2-1 therefore competed with C1q in serum for binding to CL.

Binding of purified IgA, IgG or IgM to phospholipids and their effect on C1q binding to phospholipids

Immunoglobulins from serum bind to solid-phase anionic phospholipids: for example, CL-coated wells are used in clinical assays for measuring anti-phospholipid antibodies (Harris et al., 1987). Since C1q binds to antibody-antigen complexes containing IgG or IgM, it was necessary to assess whether binding of immunoglobulin influences C1q binding. Purified human IgA, IgG and IgM were incubated with phospholipid-coated wells and to phospholipid liposomes. Tests of the quantity of IgA, IgG, IgM bound to phospholipids at saturation showed that the maximum binding of IgA and IgG from NHS was very low (0.1–16.7 fM/well) compared to

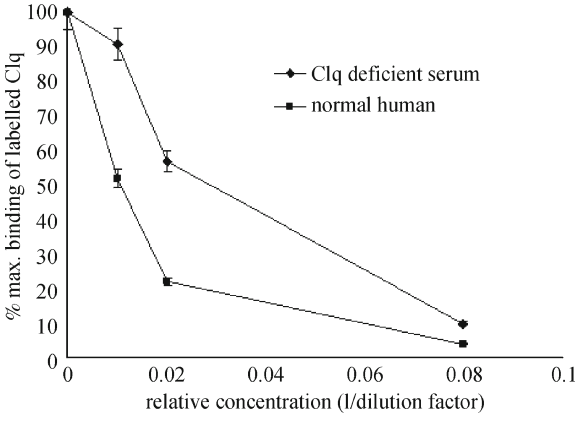

Figure 4. Inhibition of ^{125}I-C1q binding to CL-coated wells by human serum. A fixed amount of ^{125}I-C1q (10^5 cpm, 3 μg of protein) was preincubated for 1 h on ice with dilutions of normal human serum or C1q-depleted serum diluted in VB^{2+}. Mixtures were then placed in CL-coated wells for 1 h at RT. The wells were washed three times with 200 μL of VB^{2+} and the amount of bound ^{125}I-C1q was determined. Error bars represent standard deviation for three experiments.

the maximum binding of C1q or factor H (365 fM/well; Table 1). Saturation binding of immunoglobulin required a very large input of total immunoglobulin, indicating that a very low proportion of total immunoglobulin from NHS would bind to phospholipid surfaces. The low binding to CL of immunoglobulin, relative to C1q and factor H was confirmed, as shown in Fig. 3A and in Table 1.

Further experiments were carried out to determine whether the low quantity of immunoglobulin that bound to CL at saturation influenced the binding of sub-saturating quantities of C1q. A semiquantitative ELISA was used to explore the specificity of binding of the immunoglobulin, in contrast to the measurements of binding at saturation that were done using radioiodinated immunoglobulin. On phospholipid-coated wells, IgA (Fig. 5A), IgG (Fig. 5B) and IgM (Fig. 5C) all bound PA, CL, PS, PG, PI and PE. Only IgG showed significant binding to PC.

The effect of phospholipid-bound immunoglobulin on C1q binding to the phospholipid-coated wells was tested using ^{125}I-C1q (Fig. 5D–F). IgG had little effect on C1q binding (Fig. 5E). This was as expected result since the quantity of IgG bound was very low (Table 1). Furthermore, the IgG molecules were likely to be widely-spaced (the quantity of IgG shown in Table 1 would occupy only 0.01%–1.4% of the surface area of the well). IgM also had little effect on C1q binding (Fig. 5F). C1q could bind either to the phospholipids or to the bound IgM. Assuming that the affinities of C1q for phospholipids and for IgM are similar, no difference in C1q

Table 1 Saturation values for Immunoglobulin, C1q and factor H binding to phospholipids

protein coated on wells	phospholipid	protein supplied at saturation per well (fM)	amount bound per well at saturation (fM)
IgA	PA	5.3×10^3	6.4
IgA	CL	6.7×10^2	2.7
IgA	PS	5.3×10^3	5.8
IgA	PG	3.3×10^3	2.4
IgA	PI	3.3×10^3	3.7
IgA	PE	1.0×10^3	1.2
IgG	PA	5.3×10^3	1.1
IgG	CL	5.3×10^3	2.1
IgG	PS	1.0×10^4	2.0
IgG	PG	1.0×10^4	8.0
IgG	PI	1.6×10^4	3.3
IgG	PE	1.6×10^4	16.7
IgM	PA	1.1×10^2	0.1
IgM	CL	5.6×10^2	0.6
IgM	PS	1.1×10^3	0.3
IgM	PG	5.6×10^2	0.8
IgM	PI	1.1×10^2	0.2
IgM	PE	2.2×10^2	0.3
C1q	CL	6.7×10^3	366.0
factor H	CL	1.0×10^4	365.0

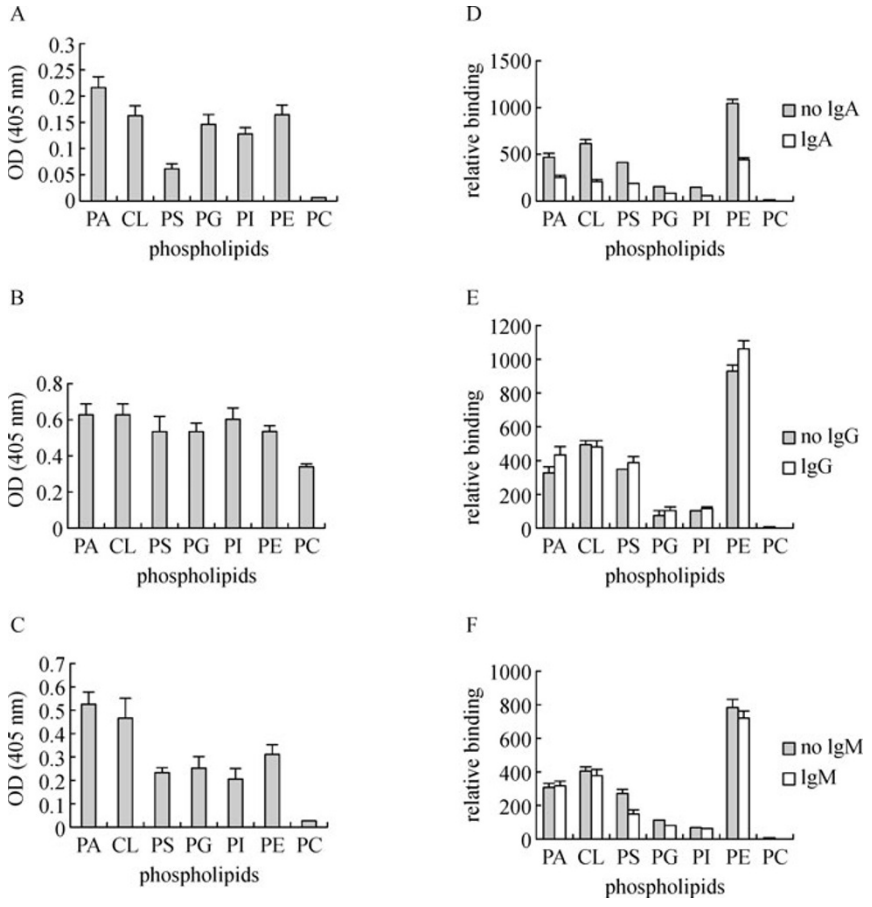

Figure 5. Binding of purified human IgA, IgG or IgM to phospholipid-coated wells and their effects on C1q binding to phospholipids. Phospholipid-coated wells were incubated with (A) purified IgA (35.7 μg /well), (B) IgG (50 μg/well) or (C) IgM (30 μg/well) in PBS-0.5mM EDTA for 1 h at RT and immunoglobulin binding was detected by use of appropriate affinity purified alkaline phosphatase-conjugated goat anti-human immunoglobulin heavy chain specific antibodies. Binding to the control wells (containing no phospholipids but blocked with ovalbumin) was negligible. The quantity of immunoglobulin used was well above the level required to achieve saturation binding (Table 1). To examine the effect of phospholipid-bound immunoglobulin on the phospholipid binding of C1q, phospholipid-coated wells were pre-incubated with (D) IgA (35.7 μg/well), (E) IgG (18.5 μg/well) or (F) IgM (20 μg/well) for 1 h at RT and the wells were then washed. A fixed amount of ^{125}I-C1q (10^4 cpm/well) in VB^{2+} was then added to all the immunoglobulin-coated wells and incubated at RT for 1 h before washing the wells and quantifying the C1q bound. Relative binding is measured as cpm bound per well. Error bars represent standard deviation for five experiments.

binding would be expected. For IgA, however, there appeared to be a consistent decrease in C1q binding (Fig. 5D). Since the quantity of IgA bound in this experiment (Table 1) was only 1.2–6.4 fM which occupied less than 0.5% of the surface area of the wells, IgA was unlikely to inhibit C1q through simple steric interference. However, the maximum binding of immunoglobulin to the phospholipid-coated wells was low and the effect on binding of C1q was small.

Purified ^{125}I-IgA, ^{125}I-IgG and ^{125}I-IgM, when incubated with phospholipid liposomes, did not show significant binding to PA, CL, PS, PG, PI, PE and PC liposomes (results not shown). Because no binding of immunoglobulin was observed, the effect of immunoglobulin on C1q binding to liposomes was not examined further.

Complement activation in NHS by phospholipids

C1q can bind to a anionic phospholipids and immunoglobulin has little influence on the binding of C1q to phospholipids. Therefore, it was of interest to investigate if phospholipids or immunoglobulin-coated phospholipid wells were capable of activating complement using a classical pathway complement consumption assay. This was done with IgG-depleted serum to further minimise possible effects of antibodies. The ionic strength of the serum incubated with phospholipid-coated wells or phospholipids liposomes was approximately 110 mM (after diluting 1:1 with $DGVB^{2+}$) compared with earlier C1q direct binding assays to PL that were performed at a salt strength equivalent to 150 mM NaCl (VB^{2+}) (Fig. 1A).

Negatively-charged phospholipids were capable of consuming complement (Fig. 6A). These included PA, CL, PS, PG and PI, all of which bear net negative charges at pH 7.4. PA and CL were more effective in consuming complement than PS, PG or PI. The neutral phospholipid, PE, could also consume complement to about the same extent as PS, PG and PI. In contrast, PC which is also net neutral at pH 7.4 failed to consume complement. Despite the low binding of immunoglobulin to phospholipid-coated wells (Table 1) and the small effect on C1q binding (Fig. 5E and 5F), presaturation of the phospholipid-coated wells with IgM or IgG does increase complement consumption. The effect of IgM was large (nearly double the consumption with PI and PE). IgG also clearly stimulated complement consumption and IgA appeared to have a regular small positive effect. Since immunoglobulin exerted only small effects on C1q binding, the influence on complement consumption was likely to be at a later stage, for example, C4 fixation (Dodds and Porter, 1979).

We also wished to examine if the alternative complement pathway was activated (consumed) by phospholipid-coated wells. There was no consumption of the alternative pathway by the seven phospholipids tested (PA, CL, PS, PG, PI, PE and PC) (Fig. 6B). The presence of bound IgG, but not IgM or IgA, stimulated very low complement consumption (4%–7%) in all the phospholipids tested except for PC where there was negligible consumption.

We examined using a classical pathway assay if phospholipid liposomes were able to consume complement. All five anionic phospholipid liposomes tested (PA, CL, PS, PG

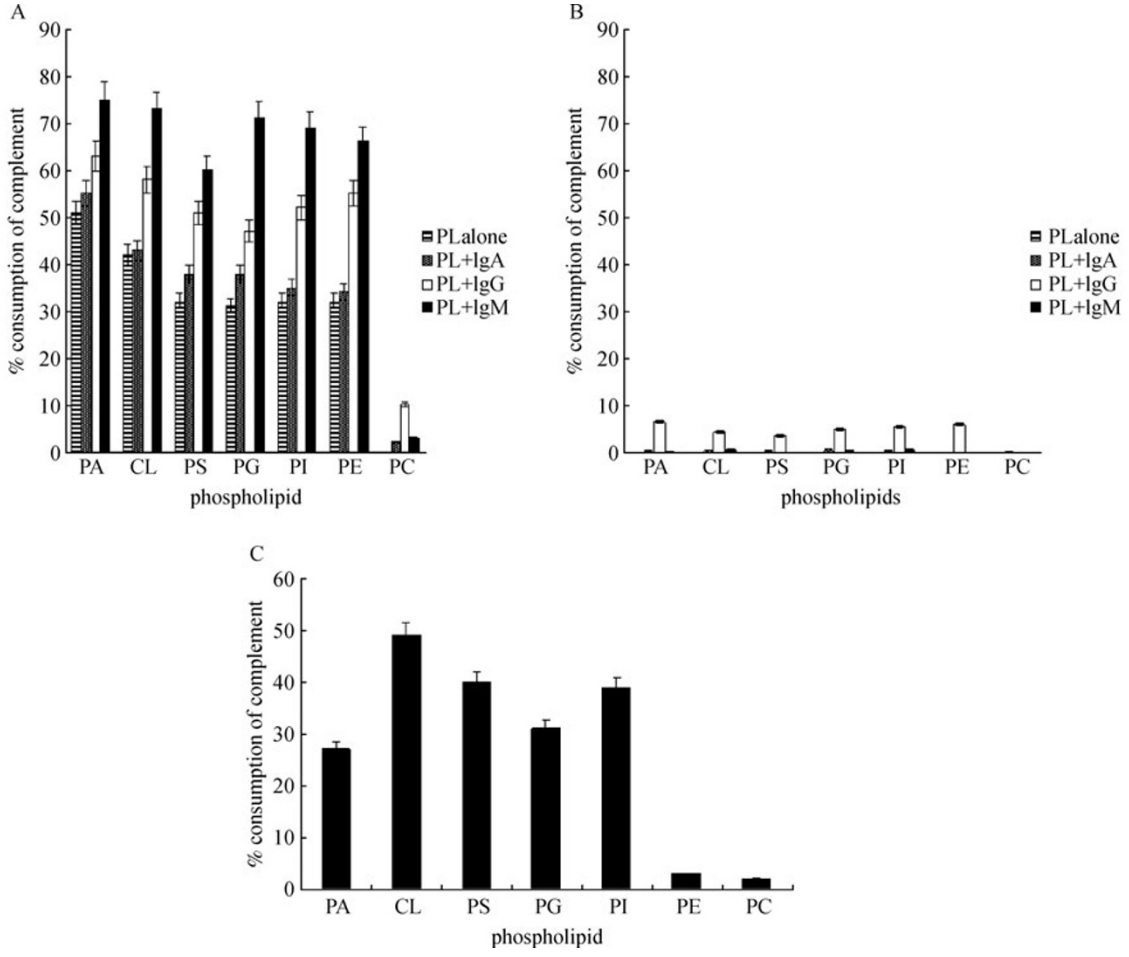

Figure 6. Complement activation in pooled human serum by phospholipids. IgG-depleted human serum was incubated with various phospholipid-coated wells or phospholipid-coated wells pre-incubated with IgA (35.7 μg/well), IgG (18.5 μg /well) or IgM (20 μg /well) for 1 h at 37°C. (A) The effect of phospholipids on the consumption of total complement or (B) alternative complement pathway was determined by complement hemolytic assays and expressed as a percentage above the baseline consumption of complement in serum incubated in control wells which were not coated with phospholipids. Pooled human serum was also incubated with various multilamellar phospholipid liposomes (C) for 1 h at 37°C. The effect of phospholipid liposomes on the consumption of total complement was also determined by complement hemolytic assays and expressed as a percentage above the baseline consumption in serum incubated with no liposomes. Error bars represent standard deviation for five experiments.

and PI) were able to consume complement (Fig. 6C) with CL showing the highest level of consumption. Neutral PE and PC liposomes did show very low consumption of complement.

Interaction of the recombinant forms of individual globular head regions of C1q with phospholipids

It has been proposed that C1q interaction with anionic phospholipids is surface charge dependent and it has been suggested it is not mediated by the globular heads (Bradley et al., 1999). We investigated if the globular heads are responsible for binding to anionic phospholipids. Using purified recombinant globular head-MBP fusion proteins from the A, B and C chains of C1q, we investigated if the globular heads were able to bind phospholipids. ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC were incubated with phospholipid-coated wells. ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC bound significantly to all the phospholipids tested except PC (Fig. 7A). The ghC module could bind to PA, CL, PS, PG, PI and PE with the highest binding observed to PA and CL. There was negligible binding of ghC to PC-coated wells. Binding of ghB to all the phospholipids was lower than for ghC, Binding of ghA was low and quite similar for all phospholipids except PC. The binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to PA and CL liposomes was also examined. All three globular head modules bound more to CL than to PA. ghA did not bind significantly to PA liposomes.

The binding of individual globular head modules and whole C1q to immobilised phospholipids were qualitatively similar, except to PE (Fig. 1 and 7A). Native C1q had very high binding to PE-coated wells but not to PE liposomes (Fig. 2). ghA, ghB and ghC showed moderate binding to PE-coated wells. It might be considered that since the recombinant heads bind less than native C1q, binding is mostly via the collagen region. However, PE-coated wells activate the classical pathway (Fig. 6A) and therefore must bind C1, in which the collagen region is mostly sterically blocked by the presence of C1r and C1s. The binding to PE-coated wells therefore may partially involve interfaces between the globular regions of different chains, e.g., A:B or A:C interfaces, which are absent in these recombinant proteins. It is likely that binding of C1q to PE-coated wells is artifactual, in that C1q interacts with a structure on PE or a relative orientation of multiple PE molecules which does not form in liposomes.

The binding of globular head modules (Fig. 7B) and whole C1q (Fig. 2) to PA and CL liposomes was also qualitatively similar.

Inhibition by C1q of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC binding to solid-phase CL

To investigate if the mode of binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to CL was similar to C1q binding, we incubated a fixed amount of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC with varying amounts of unlabelled C1q in CL-coated wells. C1q was shown to compete with all three for binding to CL (Fig. 8). C1q inhibited ^{125}I-ghA binding to CL most effectively and was less effective in competing out ^{125}I-ghB and ^{125}I-ghC. The competition suggests that the mode of binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to CL appears to be similar to that of C1q. However, < 50% inhibition was achieved with a high molar excess of C1q, so there may be additional molecular contacts with these recombinant proteins which are not present in C1q. The ovalbumin control did not interfere with the binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to CL. The

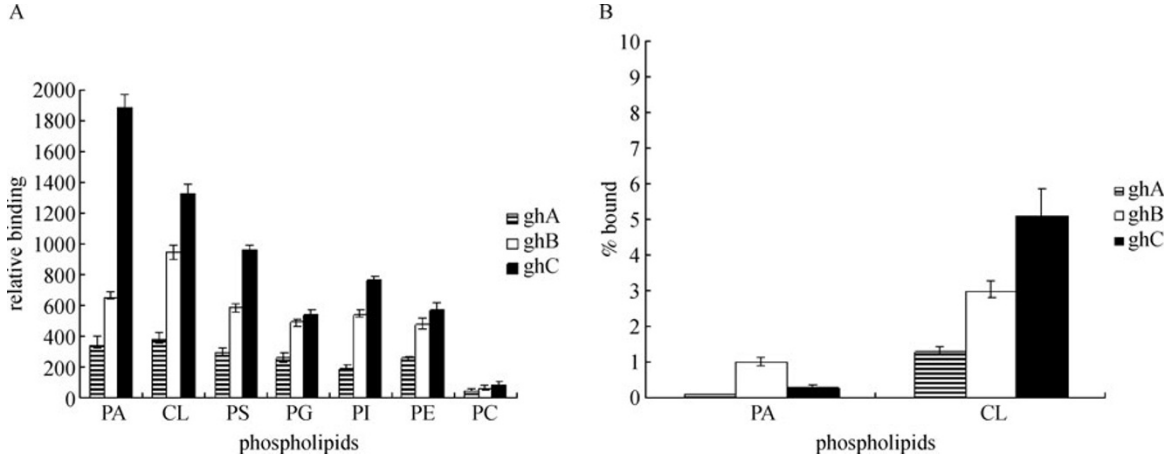

Figure 7. Binding of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to phospholipids. A: plate-bound phospholipids; B: phospholipid liposomes. The recombinant-globular heads-^{125}I-ghA or ^{125}I-ghB or ^{125}I-ghC in VB^{2+} were incubated separately with (A) solid-phase phospholipids (PA, CL, PS, PG, PI, PE or PC-coated wells) (150,000cpm/well) or (B) multilamellar phospholipid (PA, CL) liposomes (150,000 cpm/reaction) for 1 h at RT. The wells or phospholipid-pellets were washed and the amount of ghA, ghB or ghC bound was quantified. Relative binding is measured as cpm bound per well. Error bars represent standard deviation for five experiments.

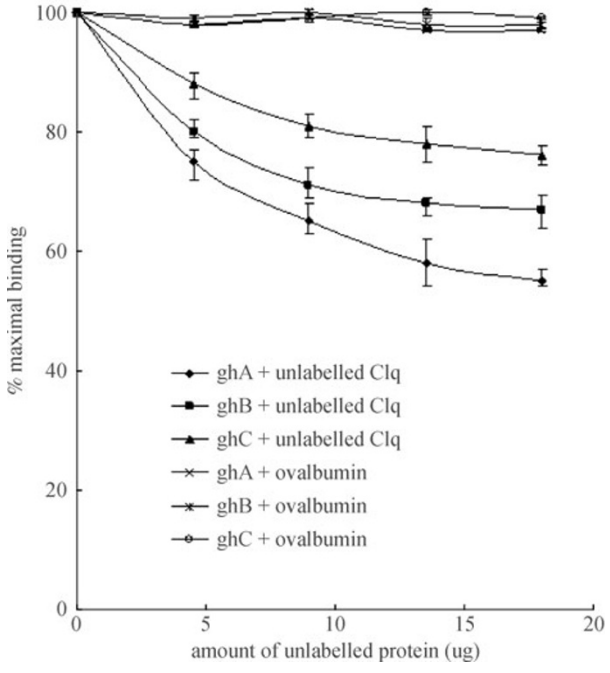

Figure 8. Inhibition of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC binding to CL-coated wells by C1q. Various amounts of unlabelled C1q (4.5–18 μg/well) were incubated with a fixed amount of ^{125}I-ghA or ^{125}I-ghB or ^{125}I-ghC (10^5 cpm/well) in CL-coated wells for 1 h at RT. Wells were washed and the amount of ^{125}I-ghA or ^{125}I-ghB or ^{125}I-ghC bound was measured. Ovalbumin was used as a control. Error bars represent standard deviation for three experiments.

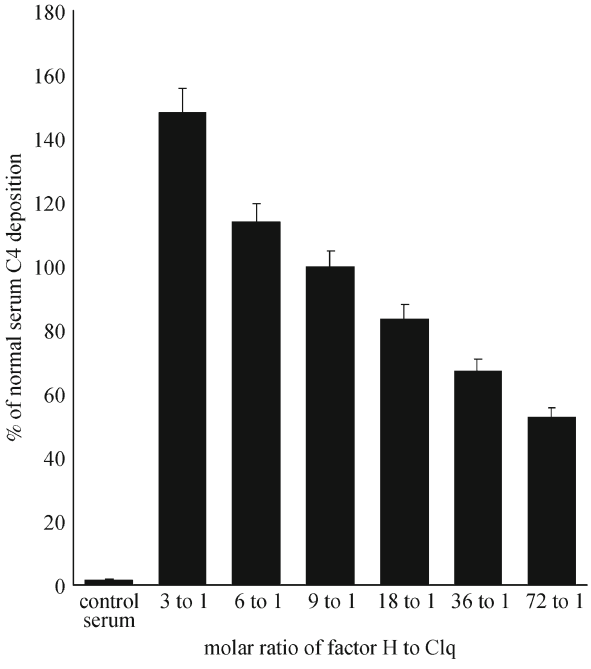

Figure 9. Influence of factor H concentration on C4 deposition on CL-coated wells. Samples of C1q and factor H-depleted serum were repleted with a constant amount of C1q and variable factor H and were incubated in CL-coated wells for 1 h at 37°C. Repletion was done by mixing 13 μL of depleted serum with 87 μL of VB^{2+} containing 0.9 μg and 0.9–21.6 μg of factor H. One hundred microlitres of the repleted serum was diluted 1:1 with $DGVB^{2+}$ and added to wells and incubated at 37°C for 1 h. The CL-coated wells were washed and the amount of C4b deposition was determined as described in Materials and Methods. The control serum is C1q and factor H depleted with no repletion. Results of three experiments are averaged.

quantity of the ghA, ghB and ghC bound in Fig. 7 are different: ghC bound best and ghA least. Similarly, as shown in Fig. 8, ghA was most readily competed out by whole C1q and ghC least readily. This may be caused by differences in the degree of oligomerisation of the ghA, ghB and ghC preparations. Gel filtration analysis (not shown) showed that the ghC contained more than 50% of material larger than trimers, ghB contained a small amount of aggregated material while ghA did not. In Fig. 7A and 7B, the results for PA and CL binding suggest that there may be some differential affinity of the different globular heads for PA and CL.

Inhibition of classical pathway activation by factor H

C1q binds by its globular heads to CL-coated wells, and this binding stimulates consumption of complement. Factor H competes with C1q for binding to CL, so factor H should inhibit classical pathway activation mediated by C1q. Since factor H, in its role as an alternative pathway downregulator, is known to affect C3 turnover, we could not use a complement activation test in which C3 turnover would influence the results. Therefore, we used C4 turnover, which precedes C3 activation, as a measure of classical pathway activation. A serum sample was depleted of C1q and factor H, and then the two proteins added back at different concentrations. The modified sera were incubated with CL-coated wells, and then the deposition of C4b was measured, as an indicator of the extent of classical pathway activation (Fig. 9). As the molar ratio of factor H:C1q increases, the deposition of C4b decreases. This shows that factor H is effectively regulating classical pathway activation.

DISCUSSION

Although complement plays an important role in the body's resistance to infectious microoganisms, it is also central to the recognition and clearance of altered or damaged host cells (apoptotic and necrotic host cells). C1q has been found to bind to subcellular particles such as mitochondria (Peitsch et al., 1988) and chromatin (Sim and Malhotra, 1994) which are exposed during cell necrosis. Many studies implicate C1q

in the uptake and clearance of apoptotic cells (Korb and Ahearn, 1997; Ogden et al., 2001). In the field of gene therapy and drug delivery systems, phospholipid liposomes have been shown to be recognized and cleared by complement (Szebeni, 1998). Charge interactions are likely to be important in the complement-mediated recognition of targets, for example, with anionic phospholipids, which are exposed on altered host cells (apoptotic and necrotic). Several studies have shown C1q binding to anionic phospholipids (Kovacsovics et al., 1985; Bradley et al., 1999) but there have been only limited studies of factor H binding to phospholipids (Kertesz et al., 1995). However, the binding of factor H to anionic surfaces for example, to synthetic polyanions has been explored (Carreno et al., 1989). Therefore, it was of interest to examine how C1q, a forward acting component and factor H, a downregulator of complement interact with phospholipids. We examined the binding of the two proteins using solid-phase bound phospholipids and multilamellar phospholipid liposomes.

^{125}I-C1q (Fig. 1) bound to all five anionic phospholipids: PG, PI, PA, PS and CL. Surprisingly, however, the highest binding was to the non-anionic PE. Binding to PC was negligible. When ^{125}I-C1q binding to phospholipids in liposomes was examined (Fig. 2), binding to all the anionic phospholipids was again observed, but there was no binding to PE liposomes. This strongly suggests that the binding of C1q to well-bound PE is an *in vitro* artifact involving forms of PE oriented differently from those that are exposed when PE is in a bilayer membrane. There was very high binding of ^{125}I-C1q to CL liposomes, while binding to PA, PS, PG and PI liposomes was lower, but above the level of the PC control.

It has been proposed that the binding of several non-immunoglobulin targets, including CL liposomes, by C1q was through the collagen region (Jiang et al., 1992, 1994; Bradley et al., 1999). In the present study, recombinant forms of globular head regions—ghA, ghB and ghC of human C1q bound to phospholipids on solid phase (Fig. 7A) and in liposomes (Fig. 7B). Comparisons between the relative binding of native C1q and recombinant globular heads to phospholipid-coated wells reveal that both native (Fig. 1) and recombinant proteins (Fig. 7A) bound PA, CL and PS, with weaker binding to PG and PI. There was no significant binding of the globular heads to PC. The recombinant globular heads bound well to CL-liposomes, and to a lesser extent, to PA-liposomes (Fig. 7B), similar to native C1q (Fig. 2). These results show that the globular heads of C1q, and not the collagen region, are responsible for interaction with anionic phospholipids (with the possible exception of the artifactual interaction with plate-bound PE). Furthermore, C1q was able to compete with the three individual globular heads for binding to CL (Fig. 8). Therefore, the mode of binding of the globular head regions is similar to that seen in whole C1q. The three C-terminal regions of C1q, A, B and C chains, have been shown to interact differentially with heat-aggregated IgG and IgM, as well as three known C1q binding peptides, derived from HIV-1, HTLV-1 and β-amyloid (Kojouharova et al., 1998; Kishore and Reid, 2000). It is notable that binding of C1q to β-amyloid fibrils, previously suggested to occur via the collagen region (A chain) (Jiang et al., 1994) has been shown to occur via the gC1q domain (Tacnet-Delorme et al., 2001).

We found that ^{125}I-factor H (Fig. 1) bound strongly to the anionic phospholipids PA, CL, PG and PI. Binding to PS was weaker, and there was negligible binding to PE and PC. The binding profile is distinct from that of C1q. Using multilamellar liposomes, factor H clearly binds to CL, while binding to PA, PS, PG and PI is low. Surprisingly, there is some interaction with PE liposomes, although there was negligible interaction with solid-phase PE. Since both C1q and factor H bind to liposomes containing CL, and also PA, PS, PG and PI, it is likely that they bind *in vivo* to cell membranes in which these phospholipids are exposed at appropriate density. C1q binding is likely to promote complement activation while factor H binding might diminish complement activation. We addressed the question whether C1q and factor H can compete for binding to immobilised phospholipids. As evident from Fig. 3B, factor H and C1q do compete for binding to immobilised CL or CL-liposomes. β_2-1 also inhibits C1q binding to immobilised CL (Fig. 3B). β_2-1 and factor H have similar binding properties to immobilised phospholipids (Yu et al., 1995, 1997; Ferluga et al., 1998). Inhibition of C1q binding to CL (on microtiter wells or in liposomes) was inhibited by 50% at a factor H/C1q molar ratio of about 1, indicating that avidity of binding is similar. Holme and colleagues (1992) suggested that factor H and C1q interact directly, so that the competition seen in Fig. 3B might result from factor H and C1q binding directly to each other, instead of the two proteins competing for binding to CL. To investigate this, binding of soluble factor H to immobilised C1q and of ^{125}I-C1q to immobilised factor H were examined. No significant binding was found. Thus, the competition between factor H and C1q for binding to CL is caused by both proteins binding to CL at the same or overlapping sites. The observations of Holme et al. (1992) on factor H-C1q interaction were made in the context of reports on RHP, a protein found in Rheumatoid Arthritis sera (Easterbrook-Smith et al., 1992; Holme et al., 1992; Hurwitz et al., 1995). RHP was reported to bind C1q and to be closely related to factor H. There was disagreement as to whether RHP bound to C1q via the globular head, or via the collagen region (Easterbrook-Smith et al., 1992; Holme et al., 1992), and also disagreement whether RHP was identical to factor H (Holme et al., 1992; Hurwitz et al., 1995). We suggest that RHP may be a post-translationally modified form of factor H with tyrosine sulphation (Sim et al., 2008). There are previous reports of two forms of factor H, Φ_1 and Φ_2 (Ripoche et al., 1988b), with distinct properties, and Φ_2, which is sulphated (Sim, R.B. and Lipscombe, R.J., unpublished) may correspond to RHP. However, our studies show that factor H from pooled normal sera does not bind C1q. It is

possible, but not examined in our study, that Φ_2 (RHP) when deposited at appropriate density, may bind C1q.

Interaction between serum and phospholipids resulted in antibody-independent activation of the classical pathway (Fig. 6). Since both C1q and factor H bind competitively to phospholipids, factor H is acting as a negative regulator of classical pathway activation. Factor H (and possibly also β_2-1) regulates classical complement pathway activation by other non-immunoglobulin activators in a similar fashion. We have evidence for this regulatory effect of factor H with several other activators (Sim et al., 2008) and Blom and colleagues have also commented on this regulatory activity (Sjöberg et al., 2009). The effect of varying factor H:C1q molar ratio on classical pathway activation in serum (Fig. 9) shows clearly the regulation of the classical pathway by factor H.

When human serum is exposed to solid-phase CL at low ionic strength (< 50 mM NaCl), IgG, β_2-1 and factor H are the major proteins which bind to CL (Yu et al., 1995; Ferluga et al., 1998). Because of the high binding of IgG under these conditions, we examined whether immunoglobulin modifies binding of C1q to phospholipids at physiologic salt. Anti-phospholipid antibodies are quite heterogenous and vary widely in quantity and specificity between different individuals. Normal individuals have low titers of antibodies against phospholipids and high levels are seen in patients with anti-phospholipid syndrome (APS). Using purified immunoglobulin from pooled NHS, we were able to show that IgG (Fig. 5B) bound to all the immobilised phospholipids while IgA (Fig. 5A) and IgM (Fig. 5C) also bound to all plate-bound phospholipids except PC. However, the quantity of immunoglobulin bound was very small (Table 1). Not surprisingly, phospholipid-bound IgG has small or negligible effect on C1q binding (Fig. 5E) given that only up to 1.4% of the surface area of the well is occupied by IgG. The area of the phospholipid-coated well was calculated from the radius and height of filling. The surface area occupied by one immunoglobulin molecule was calculated from the approximate hydrated radii of IgG, IgA and IgM (5 nm, 5 nm, 12 nm, respectively). IgM (Fig. 5F) has also very little effect on C1q binding. There was a small anomaly with IgA (Fig. 5D), which reduced C1q binding even though IgA binding occupies less than 0.5% of the surface area of the well. It is unlikely that steric interference could explain our observation. There was insignificant binding of purified immunoglobulins to phospholipid liposomes. These results show that in NHS at physiologic ionic strength, immunoglobulins do not interfere with C1q binding to phospholipids.

SDS-PAGE analysis of serum proteins binding to solid-phase CL (Fig. 3A) revealed factor H and β_2-1 as two major proteins interacting with CL. A low amount of C1q was also seen. Thus, C1q and factor H bind from whole serum, and C1q binds as C1 (since the serum used in the binding did not contain Ca^{2+} chelators). The relative quantities of C1q and factor H bound are consistent with the competition between C1q and factor H seen in Fig. 3B. In serum, there is on average approximately 8-fold molar excess of factor H over C1q (considering the concentration of factor H as 200 μg/mL and of C1q as 80 μg/mL). Taking into account β_2-1, which in this respect behaves like factor H, the molar excess of β_2-1 and factor H over C1q is about 20:1. As shown in Fig. 9, reducing the quantity of factor H relative to C1q stimulates classical pathway activation.

Despite the competition for binding to phospholipids between factor H or β_2-1 and C1q, anionic phospholipids, both solid-phase and as liposomes do activate the classical complement pathway (Fig. 6) when incubated with serum. All the anionic phospholipids (PA, CL, PS, PG and PI) on solid phase (Fig. 6A) and in liposomes (Fig. 6C) were able to activate complement. Zwitterionic PE-coated on wells, but not in liposomes, was also able to activate complement which is consistent with the observed binding of C1q to well-coated PE (Fig. 1), but not to PE liposomes (Fig. 2). Immobilised phospholipids alone did not activate the alternative pathway (Fig. 6B). These assays were done using IgG-depleted serum, and the results were consistent with direct binding of C1, and activation of C1 by the phospholipids. As suggested by Fig. 9, binding of factor H and β_2-1 diminishes the activation to the levels observed.

Measurable but very low binding (at saturation) of immunoglobulin on microtiter plates (Fig. 5, Table 1) was observed. Binding of immunoglobulin also had little effect on C1q binding (Fig. 5). Despite this, it was found that pre-saturating the immobilised phospholipids with IgG or IgM significantly increased complement consumption. In the case of pre-coating with IgM, complement consumption was increased by 50%–100% and a lesser effect was seen for IgG. IgA also had a small effect (Fig. 6A). Precoating with IgG, but not with IgA or IgM, also produced a slight consumption of alternative complement pathway activity (Fig. 6B). IgG F(ab')2 is known to activate the alternative complement pathway (Gadd and Reid, 1981).

In classical complement pathway consumption (Fig. 6A and 6C), the extent of consumption does not correlate closely with binding of purified C1q (Fig. 1 and 2). However, both factor H and β_2-1 will modulate C1q (C1) binding from serum, so the extent of activation may be a function of C1, factor H and β_2-1 binding. The complement consumption assays (Fig. 6A and 6C) were done at saturating concentrations of C1q and factor H (in serum) while the tests of isolated C1q and factor H binding to phospholipids (Fig. 1 and 2) were done below saturation, so the ratios of bound C1q/factor H at saturation cannot be judged directly from Fig. 1 and Fig. 2. The effect on complement consumption of saturating plate-bound phospholipids with immunoglobulin (Fig. 6A) is surprising, since only a very small amount of immunoglobulin is bound. IgG and IgM appear to stimulate complement activation (consumption) but do not have profound effect on C1q binding (Fig. 5). There may be several variables which

contribute to this effect: Okada et al. (1985) have suggested that C1 binds more strongly to IgG than does C1q, because of a secondary interaction between C1s and IgG Fc. Thus, IgG may have a bigger influence on C1 binding (from serum) than on binding of purified C1q. Another possible influence of immunoglobulins is in providing a suitable binding site for C4b. Dodds and Porter (1979) have shown that different C1q binding surfaces have different capacities for binding nascent C4b: for example antibody-antigen complexes fix C4b more efficiently than heat-aggregated IgG or Fc. The presence of bound immunoglobulin on the phospholipid surfaces may increase the amount of C4b2a, the classical complement pathway convertase formed.

In conclusion, this study has, for the first time, examined the interaction both of forward acting C1q and downregulatory factor H with phospholipids. C1q bound to solid-phase anionic phospholipids and in liposomes in an antibody-independent manner. The anionic phospholipids also activated complement via the classical complement pathway and immunoglobulin in normal serum has only small effects on C1q binding but has larger effects on subsequent activation of the classical complement pathway. However, factor H also binds to anionic phospholipids and acts as a direct downregulator of classical pathway activation. This activity is distinct from its well-explored role as a regulator of alternative pathway activation. This activity of factor H is likely to be important in preventing excessive (inflammatory) classical complement pathway activation by anionic phospholipids, such PS and CL, exposed on necrotic cell fragments (e.g., mitochondria (Peitsch et al., 1988)) or on cells altered by bacterial and spider venom sphingomyelinases (Tambourgi et al., 2002, 2007). PS and CL are exposed on apoptotic cell surfaces (Martin et al., 1995; Sorice et al., 2000), although they may not be the major binding sites for C1q and factor H on these cells.

MATERIALS AND METHODS

Chemicals and reagents

Inorganic salts were from Sigma (Poole, Dorset, UK), Fisons (Loughborough, Leicester, UK) or Merck/BDH Laboratory Supplies (Poole, Dorset, UK). Dithiothreitol (DTT), iodoacetamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), EDTA, polyoxyethylenesorbitan monolaurate (Tween 20), glucose, urea, (1,3,4,6)-tetrachloro-3a, 6a-diphenylglycoluril (Iodogen), Coomassie Brilliant Blue R250, p-nitrophenol phosphate, SDS polyacrylamide gel electrophoresis molecular weight markers and ovalbumin were from Sigma. Low molecular weight range prestained SDS-PAGE markers were from Gibco BRL (Gaithersburg, MD, USA). Acrylamide-bisacrylamide stock mixture for SDS-PAGE (protogel: 30% *w/v* acrylamide and 0.8% bisacrylamide) was from National Diagnostics (Hessle, Yorks, UK).

Buffers

The following buffers were used: Veronal buffers (VB, 5 mM sodium barbital, 142 mM NaCl, pH 7.4; VB^{2+}, 5 mM sodium barbital, 142 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4; $DGVB^{2+}$, dextrose gelatin veronal buffer (2.5 mM sodium barbital, 71 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% *w/v* glucose, 0.1% *w/v* gelatin, pH 7.4); DGVBMg-EGTA (7 mM $MgCl_2$, 10 mM EGTA, 21% *v/v* DGVB, 3.1% *w/v* D-glucose) and SDS-PAGE sample buffer (0.2 M Tris-HCl, 8 M urea, 2% *w/v* SDS, pH 8.0). PBS was Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 8.2 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) prepared from tablets (Oxoid: Unipath Ltd, Basingstoke, Hants, UK).

Phospholipids and antibodies

The phospholipids—PA (dipalmitoylphosphatidic acid), CL (bovine heart cardiolipin), PS (dipalmitoylphosphatidylserine), PG (dipalmitoylphosphatidylglycerol), PI (bovine liver phosphatidylinositol), PE (dimyristoylphosphatidylethanolamine) or PC (dipalmitoylphosphatidylcholine) were from Sigma as was cholesterol (CHOL). Heavy-chain-specific affinity purified secondary antibody conjugates (goat anti-human IgA alkaline phosphatase (A3063), goat anti-human IgG alkaline phosphatase (A3187), goat anti-human IgM alkaline phosphatase (A3437) and rabbit anti-mouse IgG alkaline phosphatase (A4312)) were from Sigma.

Purified proteins and antibodies

C1q was purified from pooled human serum using affinity chromatography on IgG-Sepharose. Briefly, 200 mL human plasma was made 5 mM EDTA by addition of 200 mM sodium EDTA, pH 7.4 and chilled to 0°C. IgG-Sepharose (20 mL with 20 mg of nonimmune human IgG bound/mL was added to the plasma and incubated for 45 min on ice with occasional gentle stirring. IgG-Sepharose was washed rapidly on a scintered glass funnel with ice-cold 10 mM HEPES, 140 mM NaCl, 0.5 mM EDTA, pH 7.4, until the OD_{280} of the wash was < 0.04. IgG-Sepharose was rapidly packed into a 2.5 cm diameter column, and bound C1q was eluted with a high-salt pH 11.2 buffer (e.g., 50 mM CAPS, 1 M NaCl, 5 mM EDTA). The pH of fractions was lowered by addition of 0.3 vol of 1 M sodium phosphate, pH 7, and fractions were analyzed by SDS-PAGE. Human factor H, purified by monoclonal antibody affinity chromatography (Sim et al., 1993) was provided by B.E. Moffatt, MRC Immunochemistry Unit, Oxford. Human β_2 glycoprotein-1 (β_2-1), also known as apolipoprotein H, was purified as described by Williams and Sim (1993). Human immunoglobulins (IgA, IgG and IgM) were purified from human serum pooled from at least 20 donors using published methods (Johnstone and Thorpe, 1987). The globular head regions of A (ghA), B (ghB) and C (ghC) chains were expressed in *E. coli* as fusions to maltose binding protein (MBP) and purified as described previously (Kishore et al., 2001).

Radioiodination of proteins

Iodination of immunoglobulin and factor H (Fraker and Speck, 1978)

Centrifuge tubes (1.7 mL) were coated with iodogen by evaporation of chloroform from 2 mg/mL iodogen in chloroform. Into iodogen-coated tubes (200 µg iodogen/tube) were placed 1 mCi of $Na^{125}I$ (GE Healthcare, Little Chalfont, Bucks, UK), 20 µL 1 M potassium phosphate buffer pH 7.4 and 50 µg of protein in 500 µL of PBS + 0.5 mM EDTA (PBS-EDTA). Tubes were incubated on ice for 5 min after which the solution was desalted on a PD-10 column (GE

Healthcare) with PBS-EDTA to remove unbound $Na^{125}I$. Typical specific activities obtained were 1.07×10^7 cpm/μg for IgA, 1.14×10^7 cpm/μg for IgG, 1.5×10^5 cpm/μg for IgM and 1.1×10^7 cpm/μg for factor H (counting efficiency of 70%).

Iodination of C1q, ghA, ghB and ghC

These proteins are very sensitive to oxidation and were iodinated under milder conditions. CPG-10 (BDH Chemicals, Poole, UK) controlled pore glass beads (100 mg) was mixed with 1 mL of chloroform solution containing iodogen (200 μg iodogen/mL) and incubated at RT for 5 min before the beads were dried under a stream of oxygen-free nitrogen. 10 mg of Iodogen-coated CPG-10 glass beads (20 μg iodogen/10 mg beads) were mixed with 500 μL of PBS-EDTA, 20 μL 1 M potassium phosphate buffer pH 7.4, 0.3 mCi of $Na^{125}I$ and 50 μg of protein and incubated on ice for 5 min after which the mix was transferred to a de-salting column to remove unbound $Na^{125}I$. Typical specific activities were $2.8–9.2 \times 10^5$ cpm/μg for C1q, 1.7×10^6 cpm/μg for ghA, 1.5×10^6 cpm/μg for ghB and 1.9×10^6 cpm/μg for ghC with a counting efficiency of 70%. For some experiments, radioiodinated components were diluted with unlabelled material to reduce the specific activity.

Preparation of sera

Normal human serum (NHS) was made from plasma pooled from at least 20 donors (HD Supplies, Aylesbury, UK) which was recalcified by adding 1 M $CaCl_2$ to a final concentration of 16mM and left to clot overnight at 4°C. The serum was collected by filtering through muslin.

IgG-depleted pooled human serum was made by passing 2 mL NHS through a 1 mL HiTrap protein G column (GE Healthcare) equilibrated with start buffer (20 mM potassium phosphate, pH 7.4). The sample was eluted with the start buffer at a rate of 1 mL/min.

Serum depleted of C1q was made by dialysing NHS overnight against running buffer (20 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA pH 7.4), chilling to 0°C, loading onto a Sepharose non-immune IgG column (20 mg IgG/mL) at 0°C, and eluting with ice-cold running buffer. The flow-through was tested for the absence of C1q activity by hemolytic assay. Serial dilutions in $DGVB^{2+}$ of the depleted serum (100 μL) were incubated with 1×10^7 EA cells (100 μL in $DGVB^{2+}$) at 37°C for 30 min. The EA cells were washed once by centrifugation, re-suspended in $DGVB^{2+}$ and incubated with 110 dilution of a commercial C1q-depleted serum (Sigma) in $DGVB^{2+}$ for 1 h. Lysis of EA was then measured. C1q-depleted serum was then further depleted of factor H as described by Schneider et al. (2006). Factor H depletion was measured by ELISA (Schneider et al., 2006).

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970). Sample preparation and Coomassie Blue staining was done as by Fairbanks et al. (1971).

Solid phase binding assays

Preparation of phospholipid-coated wells

Microtiter wells (96-well Maxisorp™, Nunc, Kamstrup, Denmark) were coated with 100 μL/well of 50 μg/mL of one of the phospholipids PA, CL, PS, PG, PI, PE or PC in a 2:1 (*v/v*) solution of methanol/chloroform. Solvents were evaporated in air overnight at 4°C. Wells were subsequently blocked with 200 μL of 10 mM potassium phosphate buffer, pH 7.4, and 0.5 mg/mL ovalbumin for 2 h at RT.

Preparation of protein-coated wells

Microtiter wells (Maxisorp™, Nunc) were coated with 5 μg of the sample protein (C1q or factor H) per well in 100 μL of coating buffer (0.1 M $NaHCO_3$, pH 9.6) at RT for 1 h. The wells were then washed three times with PBS containing 0.1% (*v/v*) Tween-20 (PBS-Tween) and blocked with 200 μL of PBS-Tween for 1 h at RT.

C1q and factor H binding to phospholipid-coated wells

^{125}I-C1q (10^4 cpm/well) or ^{125}I-factor H (10^4 cpm/well) was incubated in phospholipid-coated wells at RT for 1 h in a final volume of 100 μL of VB^{2+} containing 0.5 mg/mL ovalbumin. The wells were washed three times with 200 μL of VB^{2+}. Two hundred microlitres of 0.1 M NaOH was added and allowed to incubate for 5 min to dissociate bound proteins and 180 μL of the supernatant was collected and counted in a gamma-counter. In the case of binding of ^{125}I-C1q to phospholipid-coated wells pre-treated with immunoglobulin, a fixed quantity of immunoglobulin in excess of the amount needed to saturate the individual phospholipid-coated well (Table 1) was incubated for 1 h at RT with the phospholipid-coated wells (see below) The wells were washed three times as above, before ^{125}I-C1q was added and C1q binding determined as above. Wells not treated with immunoglobulin were incubated with ^{125}I-C1q as controls.

Immunoglobulin binding to phospholipid-coated wells

Dilutions of purified immunoglobulin (IgA, IgG or IgM) were placed in phospholipid-coated wells for 1 h at RT in PBS-EDTA. Following three washes with PBS-EDTA, the bound immunoglobulins were probed with affinity purified anti-immunoglobulin alkaline phosphatase conjugate in PBS-EDTA (heavy-chain specific) for 1 h at RT. After three washes in PBS-EDTA, color was developed using para-nitrophenyl phosphate (pNPP; Sigma) as a substrate, and then read at A_{405}. Controls were phospholipid-coated wells incubated with PBS-0.5 mM EDTA without immunoglobulin.

Saturation binding of immunoglobulin to phospholipids

A fixed amount of ^{125}I-immunoglobulin (10,000–50,000 cpm) (IgA, IgG or IgM) was added together with serial dilutions of unlabelled immunoglobulin (0–20 μg) in PBS-EDTA to a final volume of 100 μL and loaded onto phospholipid-coated wells (5 μg phospholipid/well), incubated for 1 h at RT and washed three times with PBS-EDTA. Quantitation of bound ^{125}I-immunoglobulin was done as for ^{125}I-C1q binding. The total amount of immunoglobulin bound was calculated from the radioactivity bound per well and the known specific activity of the dilutions.

Inhibition by human factor H and β_2-1 of ^{125}I-C1q binding to CL-coated wells

A fixed amount of ^{125}I-C1q (10^5 cpm, 3 μg of protein) was preincubated with different concentrations of unlabelled factor H or

β_2-1 for 1 h on ice in 100 µL of VB^{2+}0.5 mg/mL ovalbumin. The mixtures were placed in CL-coated wells for 1 h at RT, the wells were washed three times with 200 µL of VB^{2+} and bound ^{125}I-C1q was measured as before. Ovalbumin in place of factor H or β_2-1 was used as a negative control.

Inhibition of ^{125}I-C1q binding to CL by human serum

For the inhibition of ^{125}I-C1q binding to CL by human serum, a fixed amount of ^{125}I-C1q (10^5 cpm, 3 µg of protein) was preincubated for 1 h on ice with serial dilutions of NHS or C1q-depleted serum in VB^{2+}. Mixtures were then placed in CL-coated wells for 1 h at RT. The wells were washed three times with 200 µL of VB^{2+} and bound ^{125}I-C1q was measured as before.

Investigation of direct factor H-C1q interaction

Purified human factor H was coated on microtiter plate wells as described above. A fixed amount of ^{125}I-C1q (10^4 cpm/well) in VB^{2+}-0.5 mg/mL ovalbumin was incubated in factor H coated wells for 1 h at RT. The wells were washed three times with VB^{2+} and bound ^{125}I-C1q was detected as before. Purified C1q was coated on microtiter plate wells as described above. Factor H in PBS-Tween (5 µg/well) was incubated for 1 h at RT with the C1q coated wells. Mouse monoclonal anti-human factor H (1 µg/mL; MRC OX23) (Sim et al., 1983) and 1:7500 dilution of affinity purified rabbit anti-mouse IgG conjugated alkaline phosphatase secondary antibody were used to detect factor H. Wells coated directly with factor H were used as a positive control.

Analysis by SDS-PAGE of human serum proteins bound to CL-coated wells

Wells of a 24-well tissue culture plate; NUNC) were coated with CL (2 mL of 50 µg/mL in 2:1 (*v/v*) solution of methanol/chloroform per well). Solvents were evaporated in air at 4°C. NHS (1 mL) was diluted to 2 mL with 10 mM potassium phosphate, pH 7.4, placed in the wells and incubated at RT for 2 h. Wells were washed three times with 10 mM potassium phosphate, pH 7.4 and bound proteins were eluted with 100 µL of SDS-PAGE sample buffer (Fairbanks et al., 1971) and further analyzed by SDS-PAGE.

Binding of ^{125}I-ghA/^{125}I-ghB/^{125}I-ghC to phospholipid-coated wells

Recombinant ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC (150,000 cpm/well) were incubated in phospholipid-coated wells and binding assessed as described above for ^{125}I C1q.

Inhibition of ^{125}I-ghA/^{125}I-ghB/^{125}I-ghC binding to CL coated wells by C1q

A fixed amount of ^{125}I-ghA (10^5 cpm, 2.8 µg of protein), ^{125}I-ghB (10^5 cpm, 4 µg of protein), or ^{125}I-ghC (10^5 cpm, 1.8 µg of protein) was preincubated with different concentrations of unlabelled C1q (0–18 µg) for 1 h on ice in 100 µL of VB^{2+}-0.5 mg/mL ovalbumin. The mixtures were placed in CL-coated microtiter plate wells for 1 h at RT, the wells were washed three times with 200 µL of VB^{2+} and bound ^{125}I-ghA/^{125}I-ghB/^{125}I-ghC was measured as before. Ovalbumin in place of C1q was used as a control.

Liposome binding assays

Preparation of liposomes

Multilamellar liposomes were prepared according to established methods (New, 1990). The standard liposome preparations used in binding studies and in the functional complement activation studies were composed of 25 molar percent PC, 45 molar percent CHOL with 30 molar percent of one of the following phospholipids: PA, CL, PS, PG, PI or PE. PC liposomes contained only 55 molar percent PC and 45 molar percent CHOL. The liposome suspensions were 100 µg/mL total lipid in VB^{2+}. Quantitative determination of total phospholipids was performed using the Stewart (1980) assay.

C1q and factor H binding to phospholipid liposomes

One hundred microlitres of multilamellar phospholipid liposomes (100 µg/mL) were mixed with 200 µL of VB^{2+}-0.75 mg/mL ovalbumin containing 90,000 cpm ^{125}I-C1q or 10^5 cpm ^{125}I-factor H in 1.7 mL conical centrifuge tubes (blocked overnight with 500 µL of VB^{2+}-1 mg/mL ovalbumin) and incubated for 1 h at RT. The tubes were then spun at 10,000 g in a microfuge for 10 min and the supernatant removed. The pellet was washed once with 300 µL of VB^{2+} and spun again as before. After removing the supernatant, the pellet was counted in a gamma counter.

Immunoglobulin binding to phospholipid liposomes

Multilamellar liposomes (as above) were mixed with 20,000 cpm ^{125}I-IgA or ^{125}I-IgG or ^{125}I-IgM and the amount of immunoglobulin bound to the various phospholipid liposomes was quantified as above.

Inhibition of ^{125}I-C1q binding to CL liposomes by human factor H

^{125}I-C1q (30,000 cpm, 3 µg of C1q) was preincubated with different concentrations of unlabelled factor H for 1 h on ice in VB^{2+}-0.5 mg/mL ovalbumin. The mixtures were placed in 1.7 mL conical centrifuge tubes (blocked overnight with 500 µL of VB^{2+}-1 mg/mL ovalbumin) containing 100 µL of CL liposomes (100 µg/mL) for one hour at RT. Quantitation of the ^{125}I-C1q bound to the CL liposomes was performed as above. Ovalbumin was used as a control.

Binding of ^{125}I-ghA/^{125}I-ghB/^{125}I-ghC to PA and CL liposomes

One hundred microlitres of multilamellar PA or CL liposomes (100 µg/mL) were mixed with 200 µL of VB^{2+}-0.75 mg/mL ovalbumin containing 150,000 cpm of ^{125}I-ghA or ^{125}I-ghB or ^{125}I-ghC and binding assessed as described above for C1q and factor H.

Complement consumption assays

Preparation of sensitized sheep erythrocytes (EA) (Whaley and North, 1997)

Sheep blood in Alsevers solution (TCS Biosciences, Bucks, UK) was washed thrice in PBS before re-suspending the erythrocytes in $DGVB^{2+}$. Twenty milliliters of sheep erythrocytes (0.5×10^9/mL) was then incubated with 10 µL of antiserum (rabbit anti-sheep hemolytic serum (C12HSA, Serotec, Kidlington, UK) for 1 h at 37°C before

washing three times with $DGVB^{2+}$.

Preparation of rabbit erythrocytes (Whaley and North, 1997)

Rabbit erythrocytes were prepared from rabbit blood in Alsever's solution (TCS Biosciences) by washing rabbit erythrocytes three times in PBS before re-suspending in $DGVB^{2+}$.

Assay of total complement consumption by phospholipid-coated wells in the presence or absence of immunoglobulin

To examine complement consumption by phospholipids or phospholipid-immunoglobulin complexes, saturating amounts of immunoglobulin in a final volume of 100 µL were incubated in each phospholipid-coated well for 1 h at RT. Wells were washed 3 times with $DGVB^{2+}$. One hundred microlitres of IgG-depleted pooled NHS diluted 1:1 with $DGVB^{2+}$ was added to wells coated with phospholipids only, or phospholipids plus immunoglobulin and incubated at 37°C for 1 h. The incubated serum was serially diluted with $DGVB^{2+}$ and 100 µL of each dilution was further incubated for 1 h at 37°C with 1×10^7 (100 µL) sensitized EA to assess the remaining complement activity of the serum. The microtiter plates were then centrifuged at 2500 rpm for 15 min before collecting the supernatant and reading the absorbance at 414 nm. This assay measures the extent of complement consumption occurring during incubation with phospholipid-coated or phospholipid-immunoglobulin-coated wells by assessing the ability of the incubated serum to lyse EA. Serum incubated in wells without phospholipids but blocked with VB^{2+}-0.5 mg/mL ovalbumin was used as a control.

Assay of total complement consumption by phospholipid liposomes

Pooled NHS (200 µL) was diluted with 200 µL of phospholipid liposomes (100 µg/mL) in $DGVB^{2+}$ and incubated for 1 h at 37°C. After centrifugation (10,000 g for 10 min), the supernatant was assessed for consumption of complement as described above. Controls were serum diluted with $DGVB^{2+}$ containing no phospholipid liposomes.

Assay of alternative pathway complement consumption by phospholipid-coated wells in the presence or absence of immunoglobulin (Whaley and North, 1997)

To measure whether phospholipid surfaces or phospholipid coated with immunoglobulin (see above) can activate the alternative pathway, a 1:5 dilution of serum in DGVBMg-EGTA (7 mM $MgCl_2$, 10 mM EGTA, 21% *v/v* DGVB, 3.1% *w/v* D-glucose) was incubated with the surface for 1 h at 37°C. The serum was then serially diluted with DGVBMg-EGTA and 100 µL of the serum dilutions incubated for 1 h at 37°C with 100 µL of rabbit erythrocytes at 1×10^8/mL in microtiter wells. Subsequent steps were performed as above for assessment of classical pathway complement activity.

Inhibition of classical pathway activation by factor H

To determine the effect of factor H on C1q-initiated classical pathway activation, serum samples in which the C1q:factor H molar ratio had been adjusted to a range of values were incubated with a complement activator (CL-coated microtiter-plate wells), then the deposition of C4b on the wells was measured.

C1q and factor H-depleted serum (completely C1q deficient, but only 75% depleted of factor H) was repleted with various molar ratios of C1q and factor H and incubated in CL-coated wells for 1 h at 37°C. Repletion was done by adding a fixed quantity of C1q and a variable quantity of factor H. This was done by mixing 13 µL of depleted serum with 87 µL of VB^{2+} containing 0.9 µg of C1q and 0.9–21.6 µg of factor H. The addition of C1q restored the serum to a C1q concentration equivalent to 69ug per ml of undiluted serum. The CL-coated wells were washed 3 times in VB^{2+} and C4b fixation on the wells was measured by ELISA as described by Presanis et al. (2004).

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ABBREVIATIONS

β_2-1, β_2 glycoprotein-1; CCP, complement control protein domain; CHOL, cholesterol; CL, cardiolipin; $DGVB^{2+}$, dextrose gelatin veronal buffer (2.5 mM sodium barbital, 71 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5% *w/v* glucose, 0.1% *w/v* gelatin, pH 7.4); gC1q, the globular domain of C1q; ghA, ghB and ghC, carboxyl-terminal, globular head region of the C1q A, B and C chains, respectively expressed as MBP-fusion proteins; MBP, maltose binding protein; NHS, normal human serum; PA, dipalmitoylphosphatidic acid; PC, dipalmitoylphosphatidylcholine; PE, dimyristoylphosphatidylethanolamine; PG, dipalmitoylphosphatidylglycerol; PI, phosphatidylinositol; PS, dipalmitoylphosphatidylserine; RT, room temperature; VB^{2+}, veronal buffer (5 mM sodium barbital, 142 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4)

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