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RESEARCH ARTICLE

Interactions of complement proteins C1q and factor H with lipid A and *Escherichia coli*: further evidence that factor H regulates the classical complement pathway

Lee Aun Tan[1,2], Andrew C. Yang[3,4], Uday Kishore[5,6], Robert B. Sim[1,7] ✉

[1] Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
[2] Present address: Department of Immunology, Faculty of Medicine, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
[3] St Peter's College, New Inn Hall St., Oxford OX1 2DL, UK
[4] Present address: SUNY Downstate College of Medicine, 450 Clarkson Avenue Brooklyn, NY 11203, USA
[5] Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Headington, Oxford OX3 9DS, UK
[6] Present address: Center for Infection, Immunity and Disease Mechanisms, Biosciences, School of Health Sciences and Social Care, Brunel University, Uxbridge UB8 3PH West London, UK
[7] Present address: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK
✉ Correspondence: bob.sim@bioch.ox.ac.uk
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ABSTRACT

Proteins of the complement system are known to interact with many charged substances. We recently characterized binding of C1q and factor H to immobilized and liposomal anionic phospholipids. Factor H inhibited C1q binding to anionic phospholipids, suggesting a role for factor H in regulating activation of the complement classical pathway by anionic phospholipids. To extend this finding, we examined interactions of C1q and factor H with lipid A, a well-characterized activator of the classical pathway. We report that C1q and factor H both bind to immobilized lipid A, lipid A liposomes and intact *Escherichia coli* TG1. Factor H competes with C1q for binding to these targets. Furthermore, increasing the factor H: C1q molar ratio in serum diminished C4b fixation, indicating that factor H diminishes classical pathway activation. The recombinant forms of the C-terminal, globular heads of C1q A, B and C chains bound to lipid A and *E. coli* in a manner qualitatively similar to native C1q, confirming that C1q interacts with these targets via its globular head region. These observations reinforce our proposal that factor H has an additional complement regulatory role of down-regulating classical pathway activation in response to certain targets. This is distinct from its role as an alternative pathway down-regulator. We suggest that under physiological conditions, factor H may serve as a downregulator of bacterially-driven inflammatory responses, thereby fine-tuning and balancing the inflammatory response in infections with Gram-negative bacteria.

KEYWORDS complement, lipid A, bacteria, factor H, C1q

INTRODUCTION

The human complement system plays a major role in resistance against microbial infections via antibody-dependent and antibody-independent mechanisms. Complement is known to recognize lipopolysaccharide (LPS), which is the main amphiphilic component of the outer layer of Gram-negative bacterial membranes. LPS is typically composed of three regions: lipid A, core polysaccharide and O-specific polysaccharide (Rietschel et al., 1994). Toxicity of LPS is associated with the lipid component (lipid A) while immunogenicity is associated with the polysaccharide components. The structure of lipid A is highly conserved among eubacteria. Lipid A is composed of a β-(1→6)-linked D-glucosamine

disaccharide substituted at positions 4′ and 1 by phosphoester groups, with fatty acids linked to the remaining hydroxyl and amino groups (Rietschel et al., 1994).

LPS can activate complement via the classical, lectin and alternative pathways, resulting in the generation of pro-inflammatory anaphylatoxins (C3a, C4a and C5a) and subsequent recruitment of neutrophils and macrophages. In addition, C3b and iC3b opsonize both Gram-negative and Gram-positive bacteria (Joiner et al., 1986). The formation of the membrane attack complex and subsequent lysis of bacterial cells has been shown for a limited number of Gram-negative bacteria (Joiner et al., 1985). Opsonophagocytosis remains the major anti-bacterial defense mechanism.

LPS activates complement either in the presence of antibodies to its O-specific polysaccharide chain or by direct interaction with components of the complement system (Morrison and Kline, 1977). The lipid A region of LPS is responsible for classical pathway complement activation that involves a direct interaction between lipid A and C1q leading to the activation of C1 (Cooper and Morrison, 1978; Arvieux et al., 1984; Loos and Clas, 1987; Clas et al., 1989). The polysaccharide region, according to its composition, may stimulate alternative and lectin pathway activation through lipid A-independent mechanisms (Morrison and Kline, 1977; Jack and Turner, 2003). We have recently found that C1q and factor H bound to immobilized as well as liposomal anionic phospholipids, most notably to cardiolipin. Factor H strongly inhibited C1q binding to solid phase cardiolipin and cardiolipin liposomes, suggesting a role for factor H in regulating activation of the classical complement pathway by anionic phospholipids (Tan et al., 2010). In the work presented here, we investigated whether lipid A, a well-established classical pathway activator, also interacts with factor H as well as C1q, and followed this by tests of binding to a laboratory strain of *E. coli* as a source of lipid A.

Complement activation by the classical pathway occurs when the first component of complement, C1, binds via charge interactions to immune complexes containing IgG or IgM or directly to non-immunoglobulin target surfaces such as anionic phospholipids and Gram-negative bacteria (Sim and Malhotra, 1994; Gaboriaud et al., 2003; Kojouharova et al., 2004; Kishore et al., 2004a: Roumenina et al., 2006; Tan et al., 2010). C1 is made up of three glycoproteins, C1q, C1r and C1s. C1r and C1s are serine proteases that become activated when C1q binds to a target. C1q is a 460 kDa glycoprotein composed of 18 polypeptide chains (6A, 6B and 6C) and is present in human serum at a concentration of about 115 mg/L (Reid and Porter, 1976; McAleer and Sim, 1993; Dillon et al., 2009). The A, B and C chains, each about 25 kDa, combine to form six equivalent subunits each with a collagen-like “stalk” at the amino-terminus and a carboxyl-terminal heterotrimeric globular (gC1q) domain. The six subunits associate to form an umbel shape. The C1r and C1s subcomponents of C1 bind the collagen region of C1q. Binding of C1q to ligands, which occurs via the gC1q domains, brings about a conformational change in the collagen region that activates C1r and C1s (Dodds et al., 1978).

Factor H is a single polypeptide chain protein of 155 kDa, composed of 20 complement control protein (CCP) modules (Ripoche et al., 1988). Its concentration in human sera is reported to vary widely between individuals (Charlesworth et al., 1979; Maruvada et al., 2008; Edey et al., 2009; Ingram et al., 2010) up to over 800 mg/L, but probably on average 200 mg/L (Sim et al., 1993). It is well-known as a regulator of the complement alternative pathway. 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 (Weiler et al., 1976; Whaley and Ruddy, 1976; Fearon, 1978; Sim et al., 1993). The discrimination between activators and non-activators of the alternative pathway is determined by binding of factor H to surface-associated C3b (Meri and Pangburn, 1990). Factor H binds directly to many microorganisms and parasites where it is thought to downregulate complement alternative pathway activation (Díaz et al., 1997; Kraiczy and Würzner, 2006).

Since both C1q and factor H have crucial roles in the recognition and removal of bacteria, we examined whether factor H might regulate C1q binding to lipid A, thus limiting the level of the activation of the classical pathway. The study was undertaken with lipid A coated on wells and incorporated into liposomes. The binding characteristics of both complement proteins to a Gram-negative bacterium, *Escherichia coli* strain TG1, were also investigated.

RESULTS

Interaction of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC with lipid A

Purified radioiodinated proteins were incubated in microtiter wells coated with lipid A. C1q bound strongly to lipid A-coated wells, with lower binding by factor H and ghC (Fig. 1a). Lowest binding to lipid A was seen with ghA and ghB but this was still above the background levels observed with negative control wells coated with phosphatidylcholine (PC). Cardiolipin (CL)-coated wells were used as a positive control (Tan et al., 2010).

Lipid A liposomes were also incubated with radioiodinated proteins and the binding was assessed (Fig. 1B). Factor H, ghA and ghC showed strongest binding to lipid A liposomes. The binding of C1q and ghB was less but well above background binding levels observed to control PC liposomes. CL liposomes were used as a positive control. These results show that C1q and factor H bind to lipid A and that the recombinant globular head regions of C1q bind differentially to lipid A. As noted previously (Tan et al., 2010), the oligomerization state of the recombinant gHA, gHB, gHC proteins varies, although they are mostly in trimer form. These materials may exhibit higher, but more usually lower, binding

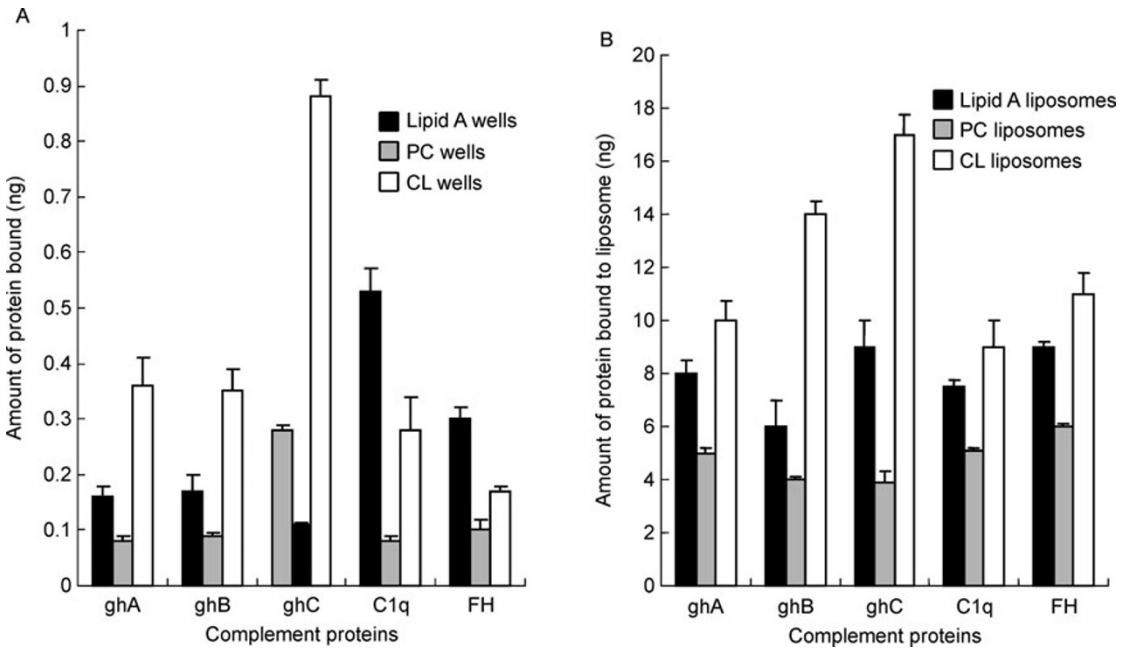

Figure 1. Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to lipid A, PC and CL-coated wells and liposomes. (A) Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to lipid A, PC and CL coated wells. A fixed amount of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB, and ^{125}I-ghC was incubated with lipid A-coated wells (5 μg lipid A/well) for 1 h at room temperature in VB^{2+}. The wells were washed, and the amount of bound C1q, factor H, ghA, ghB, and ghC measured. Cardiolipin (CL) served as a positive control while phosphatidylcholine (PC) served as a negative control. Error bars represent standard deviation for five experiments. (B) Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB, and ^{125}I-ghC to lipid A, PC and CL liposomes. A fixed amount of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC was incubated with lipid A liposomes (10 μg/reaction) for 1 h at room temperature in VB^{2+}. The liposomes were washed, and the amount of bound C1q, factor H, ghA, ghB and ghC measured. CL served as a positive control while PC served as a negative control. Error bars represent standard deviation for five experiments.

affinity than C1q, depending on polymerization state. They may possess potential binding sites not present in native C1q, because of different domain-domain interfaces.

Saturation binding of ^{125}I-C1q to lipid A

A fixed amount of ^{125}I-C1q was incubated with increasing amounts of unlabelled C1q on lipid A-coated wells or lipid A liposomes. The C1q binding to lipid A-coated wells (Fig. 2) and lipid A liposomes (Fig. 2) was dose dependent and saturable. ^{125}I-C1q binding to lipid A-coated wells and lipid A liposomes was saturated at a final concentration of 10 μg/mL C1q, which is well below the plasma concentration of ~115 μg/mL.

Inhibition of ^{125}I-C1q binding to lipid A by factor H

Since both C1q and factor H bound to lipid A, we examined whether they competed with each other. On lipid A-coated wells, a 6.75-fold molar excess of unlabelled factor H over ^{125}I-C1q was able to inhibit C1q binding by 40% (Fig. 3A). Higher amounts of factor H diminished C1q binding by > 80%. The molar ratio of factor H to C1q in serum may vary between about 5:1 and 30:1. Ovalbumin (OVA) did not influence the binding of ^{125}I-C1q to lipid A-coated wells.

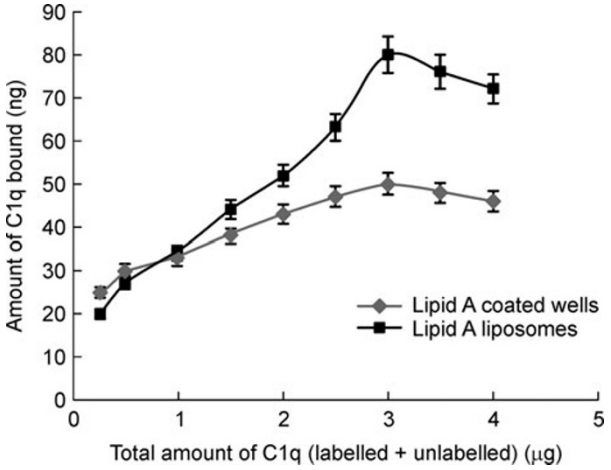

Figure 2. Saturation of ^{125}I-C1q binding to lipid A-coated wells and lipid A liposomes. A fixed amount of ^{125}I-C1q (250 ng per well or per reaction) was mixed with increasing amounts of unlabelled C1q (0–3.75 μg per well or per reaction) in VB^{2+} and loaded onto lipid A-coated wells or mixed with lipid A liposomes. Saturation of ^{125}I-C1q binding to lipid A was seen at an input of about 3 μg of C1q. Error bars represent standard deviation for three experiments.

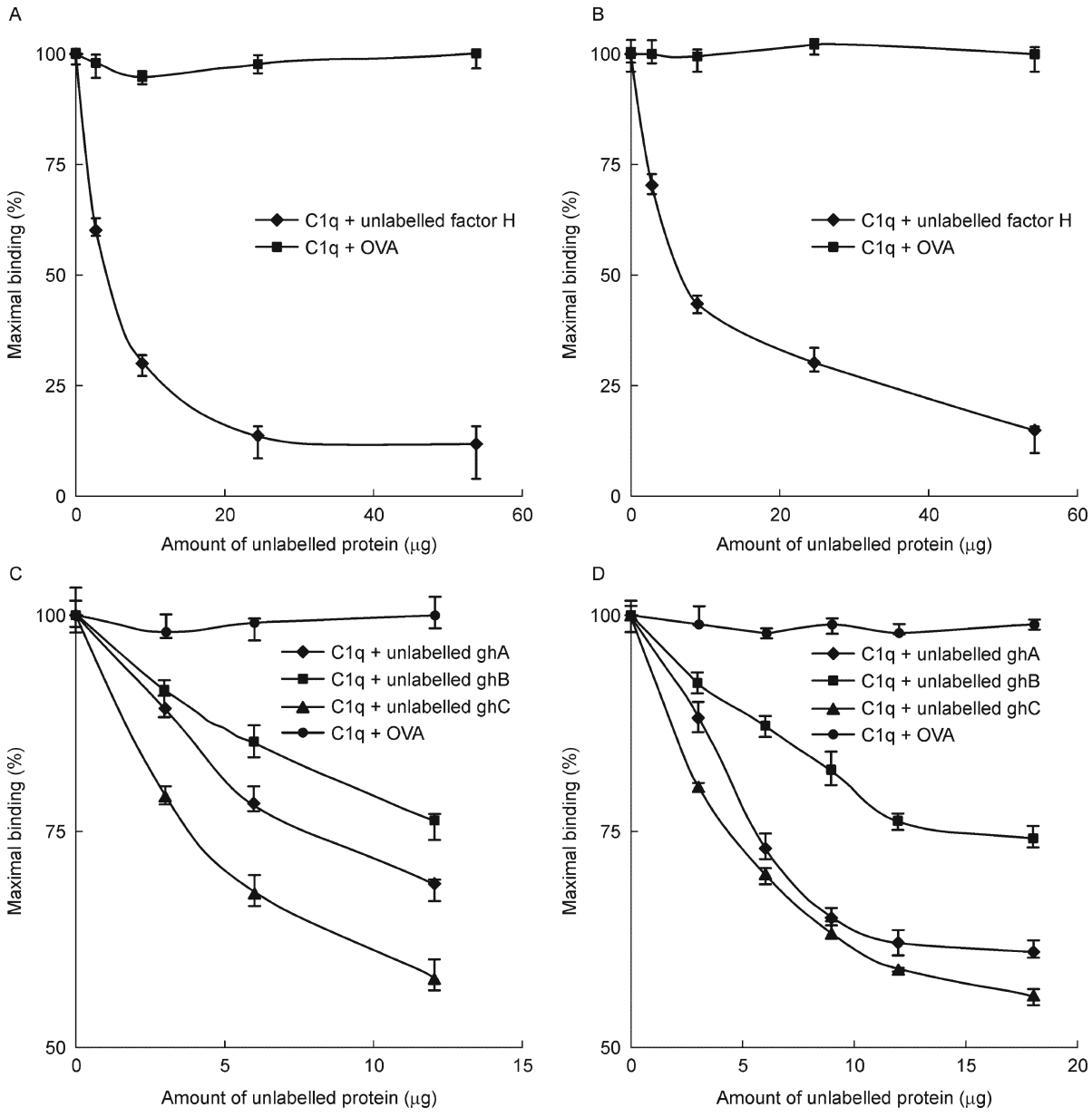

Figure 3. Inhibition of ^{125}I-C1q binding to lipid A by factor H or by ghA, ghB, and ghC. A fixed amount of ^{125}I-C1q in VB^{2+} (3 μg per well or per reaction) was incubated with various quantities of unlabelled factor H (0–54 μg) either on lipid A-coated wells (A) or with lipid A liposomes (B) at room temperature for 1 h. The wells or liposomes were washed and the amount of bound C1q was determined. Ovalbumin (OVA) was used as a control protein. Error bars represent standard deviation for three experiments (1 μg of factor H is the molar equivalent of 3 μg C1q). Similarly, a fixed amount of ^{125}I-C1q in VB^{2+} (3 μg per well or per reaction) was incubated with various quantities of unlabelled ghA, ghB and ghC (0–18 μg) either on lipid A-coated wells (C) or with lipid A liposomes (D) at room temperature for 1 h. The wells or liposomes were washed and the amount of bound C1q was determined as above.

When ^{125}I-C1q was incubated with 6.75-fold molar excess of factor H and mixed with lipid A liposomes, there was 30% decrease in C1q binding to lipid A liposomes (Fig. 3B) and again higher amounts of factor H diminished C1q binding by about 80%. OVA again did not interfere with the binding. These results indicate that C1q and factor H compete for binding to lipid A.

C1q binds to lipid A via its globular heads

The recombinant ghA, ghB and ghC modules were shown to bind to both lipid A-coated wells (Fig. 1A) and lipid A

liposomes (Fig. 1B). To ascertain if the native C1q bound lipid A via its gC1q domain, ^{125}I-C1q was incubated with unlabelled ghA, ghB or ghC on lipid A-coated wells and lipid A liposomes.

C1q binding to lipid A-coated wells was inhibited most strongly by ghC (Fig. 3C) with weaker inhibition by ghA and ghB. The binding of C1q to lipid A liposomes was also strongly competed out by ghC and ghA with weaker inhibition by ghB (Fig. 3D), confirming that C1q binds to lipid A via its gC1q domain. The ghC module appeared to be the strongest lipid A binder (Fig. 1A and 1B), consistent with the inhibition assays where ghC was most effective in competing out C1q binding to lipid A-coated wells (Fig. 3C) and lipid A liposomes (Fig. 3D). As shown in Fig. 3C and 3D, the competition was incomplete since < 50% inhibition was achieved at the highest ghA, ghB and ghC concentrations used. This may be associated with the number of binding sites per molecule on C1q, compared with the ghA, ghB and ghC modules. C1q is likely to engage up to 18 binding sites, while ghA, ghB and ghC have, in principle, 3 sites each. The binding of C1q should therefore be of higher avidity, and the kinetics of binding may also be different. Alternatively, there may be a contribution of the C1q collagen region to the binding.

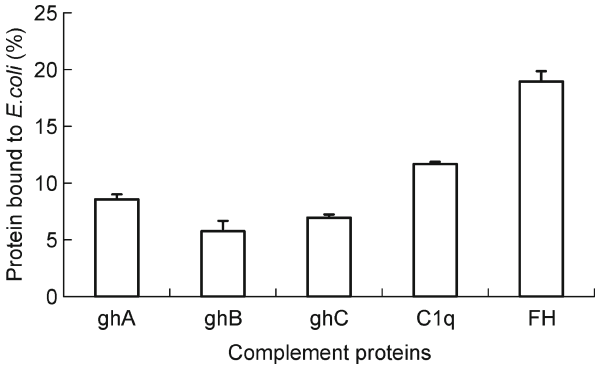

Figure 4. Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to *E. coli* TG1. One hundred microliters of VB^{2+}-0.5 mg/mL ovalbumin containing 125 ng of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC was incubated with 200 µL of *E. coli* (1 × 10^9 cells/mL in VB^{2+}-0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+}-1 mg/mL ovalbumin) and incubated at 37°C for 1 h with mixing every 15 min. Cells were centrifuged and then the radioactivity was counted. Error bars represent standard deviation for five experiments.

Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to *E. coli* TG1

Since all these proteins bind to lipid A isolated from *E. coli*, we tested the binding to the whole bacteria. Radioiodinated C1q, factor H, ghA, ghB and ghC did bind to the bacterial cells (Fig. 4). Factor H especially showed high binding to the bacterial cells, with approximately 20% of the protein incubated binding to the cells (Fig. 4). C1q binding was about 12% while the recombinant globular head regions showed 6%–8% binding to the bacterial cells. Relative binding by the different proteins is similar to that seen with lipid A liposomes (Fig. 1B), suggesting that binding to lipid A may be a major contributor to the total binding observed.

Saturation binding of ^{125}I-C1q and ^{125}I-factor H to *E. coli*

When ^{125}I-C1q or ^{125}I-factor H was incubated with their respective unlabelled counterparts together with the bacterial cells, we observed that C1q and factor H binding to *E. coli* was dose dependent. C1q binding was saturable at 550 ng of C1q (approximately 2 µg/mL final concentration) (Fig. 5A) while the saturation of factor H binding to *E. coli* was observed at an input of 800 ng (approximately 2.7 µg/mL) (Fig. 5B). The numbers of molecules per cell bound at saturation correspond to about 400 for C1q, and 1000 for factor H.

Specific binding of ^{125}I-C1q and ^{125}I-factor H to *E. coli*

Since factor H and C1q binding to *E. coli* was saturable, we examined if the binding of both proteins to *E. coli* was specific. Inhibition of ^{125}I-C1q with unlabelled C1q, using OVA as a control, was first studied. At a molar ratio of unlabelled C1q to labelled C1q of 4:1, ^{125}I-C1q binding was inhibited by about 50% (Fig. 5C). Thus, the radioiodinated C1q bound slightly better than unlabelled C1q, suggesting limited modification of binding properties during iodination (for example, by oxidation). Alternatively, specific binding may be superimposed on a significant non-specific component. OVA did not inhibit ^{125}I-C1q binding to *E. coli*.

When the specificity of ^{125}I-factor H binding to bacterial cells was examined, a 5:1 molar ratio of unlabelled factor H to labelled factor H inhibited ^{125}I-factor H binding by about 50% (Fig. 5D). Again this may indicate some damage to the protein on radioiodination or a non-specific component to the binding, as for C1q. OVA did not interfere with ^{125}I-factor H binding. These results show that the binding of C1q and factor H to *E. coli* have specific and saturable components, but there may be a significant background of non-specific binding.

Saturation binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to *E. coli*

^{125}I-ghA, ghB and ghC were incubated with various quantities of unlabelled ghA, ghB and ghC respectively and it was shown that all three proteins bound to *E. coli* in a dose-dependent manner. Saturation was observed at an input of 1.55 µg for ghA (Fig. 6A), 2.05 µg for ghB (Fig. 6B) and 3.05 µg for ghC (Fig. 6C). Saturation required a higher quantity of these proteins than for C1q (0.55 µg), consistent with probable difference in avidity as discussed above.

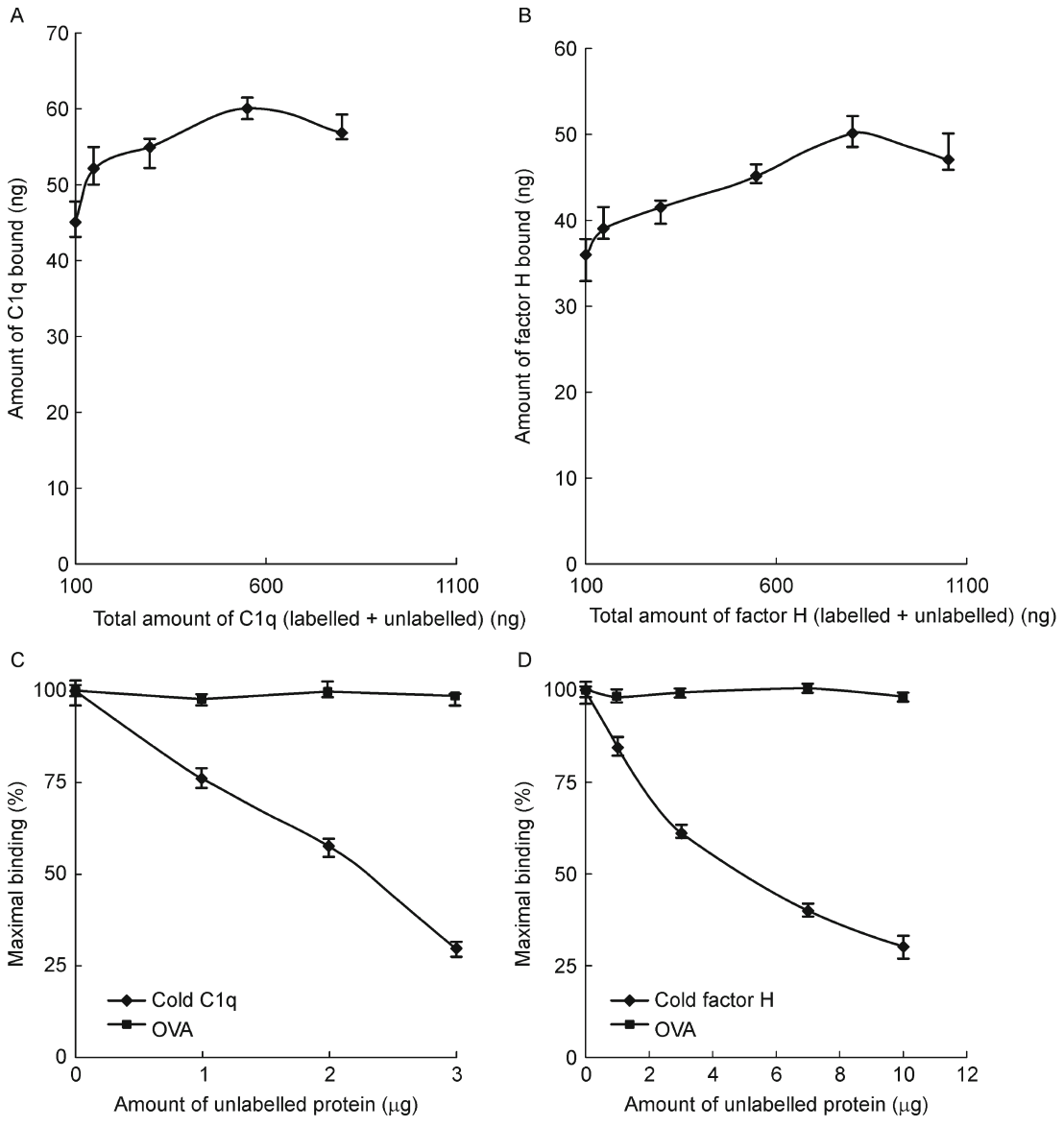

Figure 5. Saturation and specificity of binding of ^{125}I-C1q or ^{125}I-factor H to *E. coli*. For saturation binding, ^{125}I-C1q (50 ng/reaction) (A) was mixed with varying quantities of unlabelled C1q in 100 μL of VB^{2+}-0.5 mg/mL ovalbumin for 1 h on ice. The mixture was then incubated with 200 μL of *E. coli* (1×10^9 cells/mL in VB^{2+}-0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes at 37°C for 1 h with mixing every 15 min. Similarly, in (B) ^{125}I-factor H (50 ng/reaction) was mixed with varying quantities of unlabelled factor H in 100 μL of VB^{2+}-0.5 mg/mL ovalbumin for 1 h on ice. The reaction mix was then incubated with *E. coli* as above. Cells were centrifuged and then bound radioactivity was counted. Error bars represent standard deviation for three experiments. To test specificity, for C1q (C) a fixed amount of ^{125}I-C1q (55 ng C1q/reaction) was preincubated with different quantities of unlabelled C1q (0–3 μg) with ovalbumin as control for 1 h on ice. Mixtures were then incubated with *E. coli* (2×10^8 cells/reaction) for 1 h at 37°C and binding was assessed. For factor H (D) a fixed amount of ^{125}I-factor H (80 ng/reaction) was preincubated with different quantities of unlabelled factor H (0–10 μg/reaction) with ovalbumin as control for 1 h on ice. Mixtures were then incubated with *E. coli* (2×10^8 cells/reaction) for 1 h at 37°C and binding was measured. Error bars represent standard deviation for five experiments.

Specific binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to *E. coli*

Labelled ghA, ghB and ghC were incubated with their respective unlabelled counterpart and the binding of ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to *E. coli* was assessed. A 5:1 molar ratio of ghA to labelled ghA yielded about 50% decrease in ^{125}I-ghA binding (Fig. 6D) and similar results were obtained for ghB and ghC. OVA did not inhibit the binding of all three labelled proteins to *E. coli*. These results demonstrate that

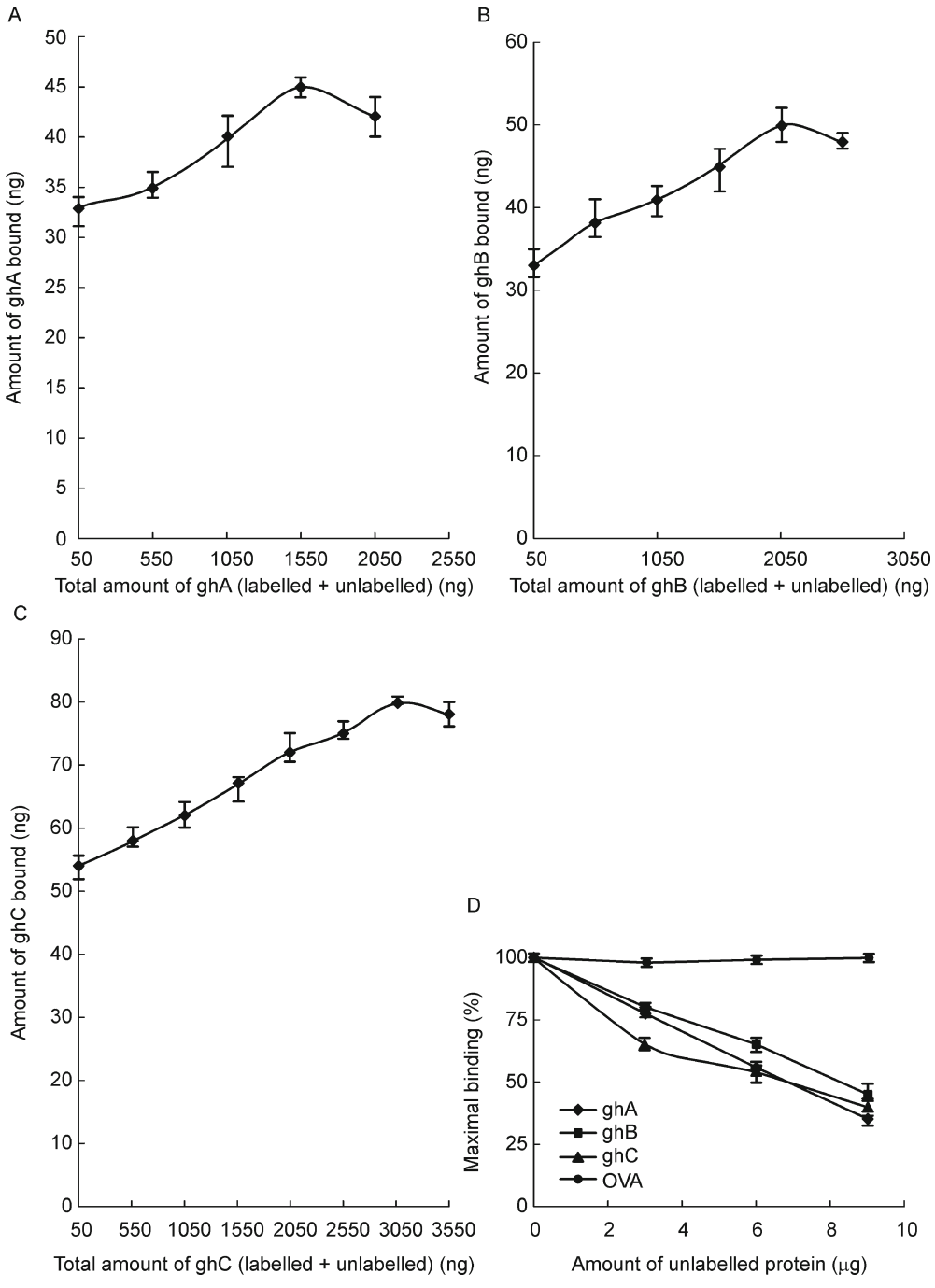

Figure 6. Saturation and specificity of binding of ^{125}I-ghA, -gh-B, -gh-C to *E. coli*. ^{125}I-ghA (A) or ^{125}I- gh-B (B) or ^{125}I-gh-C (C) (50 ng/reaction) were mixed with varying quantities of their unlabelled counterparts in 100 μL of VB^{2+}-0.5 mg/mL ovalbumin for 1 h on ice. The reaction mixtures were then incubated with 200 μL of *E. coli* (1×10^9 cells/mL in VB^{2+}-0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 μL of VB^{2+}-1 mg/mL ovalbumin) at 37°C for 1 h with mixing every 15 min. Cells were centrifuged and then the radioactivity was counted. Error bars represent standard deviation for five experiments. To test specificity of binding of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to *E. coli* (D), ^{125}I-ghA (1.55 μg), or ^{125}I-ghB (2.05 μg) or ^{125}I-ghC (3.05 μg) was incubated with increasing quantities of the corresponding unlabelled ghA, ghB and ghC respectively (or with the negative control, ovalbumin (OVA) in 100 μL of VB^{2+}-0.5 mg/mL ovalbumin and incubated on ice for 1 h. The mix was added to 200 μL of *E. coli* (1×10^9 cells/mL in VB^{2+}-0.5 mg/mL ovalbumin) in 1.7 mL eppendorf tubes (blocked overnight with 500 μL of VB^{2+}-1 mg/mL ovalbumin) and incubated at 37°C for 1 h with occasional mixing every 15 min. Cells were centrifuged and bound radioactivity was measured. Error bars represent standard deviation for five experiments.

ghA, ghB and ghC binding to *E. coli* have a specific and saturable component.

C1q and factor H compete for binding to *E. coli*

Since both C1q and factor H bind to *E. coli* in a specific and saturable manner, we extended our study to whole bacterial cells in order to compare our results with immobilized and liposome lipid A (Fig. 3A and 3B). When ^{125}I-C1q was mixed with unlabelled factor H, there was partial inhibition of C1q binding to *E. coli*. When a 9:1 molar ratio of unlabelled factor H to C1q was incubated (1.66 μg of factor H), there was about 20% inhibition of ^{125}I-C1q binding (9:1 is within the range of molar ratios found in plasma) (Fig. 7A). At the highest quantity of factor H used (approx 40:1 molar ratio to C1q) inhibition approached 50%.

Unlabelled C1q also competed with ^{125}I-factor H for binding (Fig. 7B) and about 25% inhibition was seen with a 1:1 C1q to factor H molar ratio (2.4 μg C1q). Inhibition approached 50% at the highest C1q concentration used (15 μg C1q, molar ratio C1q : factor H = 6.3:1). Since the relative number of binding sites and their affinities differ for C1q and factor H, it is possible that different incubation times would give a different binding profile. The OVA control did not interfere with ^{125}I-C1q or ^{125}I-factor H binding to bacterial cells. These results demonstrate that C1q and factor H do compete for binding to *E. coli* but only partial inhibition was achieved. While factor H and C1q share some binding sites on bacterial cell surfaces, there are likely to be other sites on *E. coli* that are uniquely bound by either factor H or C1q.

Assessment of C4b deposition on lipid A-coated wells

Since factor H inhibited C1q binding to lipid A-coated wells and liposomes, it was of interest to evaluate if this was of significance in the context of regulation of classical complement pathway activation. We used factor H- and C1q-depleted serum repleted with various C1q and factor H ratios, incubated on lipid A-coated wells. The extent of activation of the classical pathway was assessed by measuring C4b bound to the wells. We observed a trend that an increase in the factor H concentration decreases the amount of C4b deposition (Fig. 8). These results are consistent with a regulatory role for factor H in down-regulating classical complement pathway activation.

DISCUSSION

LPS is the major component of the outer membrane of Gram-negative bacteria and a well known activator of the humoral

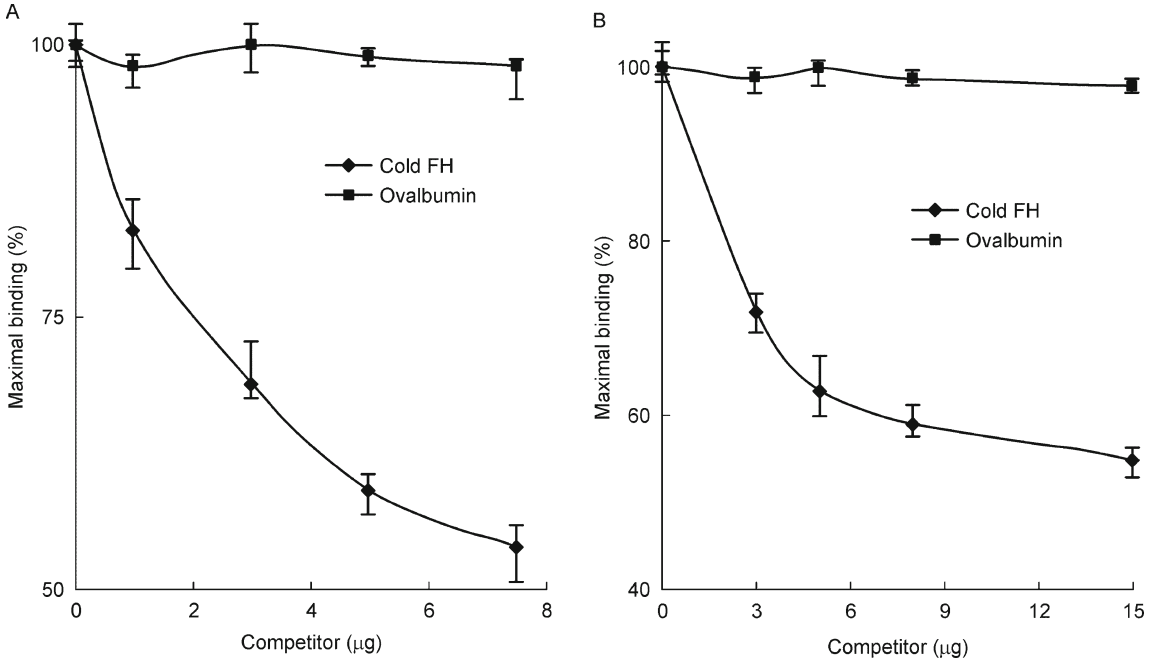

Figure 7. Reciprocal inhibition of ^{125}I-C1q and factor H binding to *E. coli*. (A) Inhibition of ^{125}I-C1q binding to *E. coli* by factor H. A fixed amount of ^{125}I-C1q in VB^{2+} (0.55 μg C1q/reaction) was incubated with various quantities of unlabelled purified factor H (0–7.5 μg/reaction) and mixed with *E. coli* (2×10^8 cells/reaction) for 1 h at 37°C. Cells were centrifuged and bound radioactivity was measured (0.185 μg of factor H is the molar equivalent of 0.55 μg of C1q). (B) Inhibition of ^{125}I-factor H binding to *E. coli* by C1q. Conversely, a fixed amount of ^{125}I-factor H in VB^{2+} (0.80 μg factor H/reaction) was incubated with various quantities of unlabelled C1q (0–15 μg/reaction) and mixed with *E. coli* (2×10^8 cells/reaction) for 1 h at 37°C. Cells were centrifuged and bound radioactivity was measured (2.40 μg C1q is the molar equivalent of 0.80 μg factor H). In both experiments, Ovalbumin (OVA) was used as a control protein. Error bars represent standard deviation for three experiments.

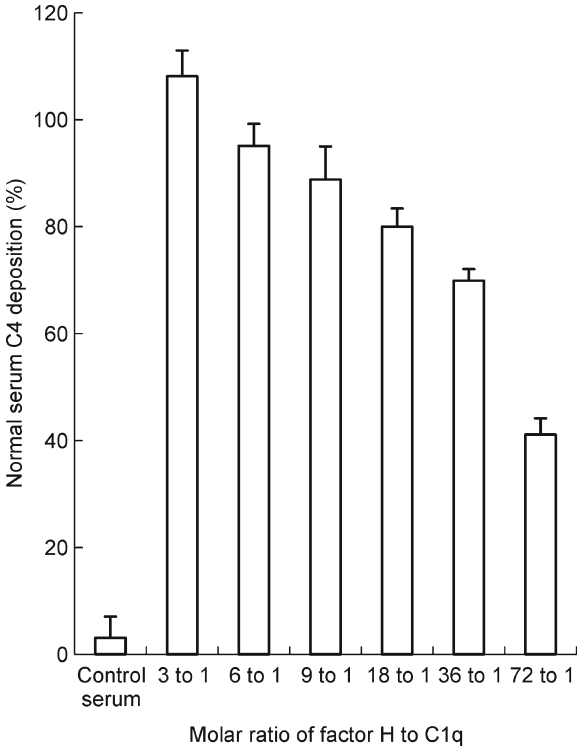

Figure 8. Assessment of C4 deposition on lipid A-coated wells. C1q and factor H-depleted sera, repleted with various molar ratios of C1q and factor H, were incubated in lipid A-coated wells for 1 h at 37°C. The depleted serum is essentially completely C1q-deficient, but only 75% factor H depleted. Repletion was done by adding a fixed quantity of C1q and a variable quantity of factor H to the depleted serum. This was achieved by mixing 13 μL of depleted serum with 87 μL of VB^{2+} containing 0.9 μg C1q and 0.9–21.6 μg of factor H. The lipid A-coated wells were washed and the relative amount of C4 deposition was determined. Error bars represent standard deviation for five experiments. "Control serum" is the depleted serum with no addition of C1q or factor H.

and cellular components of the host defense system (Rietschel et al., 1994). Activation of host immune mechanisms, including the complement system, is essential to fight infection with Gram-negative bacteria, although excessive stimulation can also result in the serious-life threatening symptoms of septic shock (Bone 1991). LPS activates complement either in the presence of antibodies to its O-specific polysaccharide chain or by direct interaction with components of the complement system (Morrison and Kline, 1977). They first demonstrated that the lipid A region of LPS is responsible for classical pathway complement activation while the polysaccharide region is responsible for alternative pathway activation through a lipid A-independent mechanism. More recent data indicate that the polysaccharide regions may also activate the lectin pathway (Jack and Turner, 2003; Shang et al., 2005). Direct interaction of isolated lipid A with C1q leading to the activation of C1 was reported by Cooper and Morrison (1978).

We have studied interactions of C1q and factor H with lipid A purified from *E. coli* and also intact *E. coli* cells. C1q was shown to bind to lipid A coated on wells (Fig. 1A and 1B) and in liposomes. The binding of C1q is predominantly via its globular heads (Fig. 1A, 1B, 3A and 3D). The observation of factor H binding to both forms of lipid A is novel (Fig. 1A and 1B). Since C1q and factor H both bind to lipid A, we examined if factor H competes with C1q for anionic binding sites like those presented by lipid A. As illustrated in Fig. 3A and 3C, unlabelled factor H does effectively compete with ^{125}I-C1q binding to lipid A. This novel observation suggests that factor H has an additional regulatory role of down-regulating classical pathway activation. This was confirmed (Fig. 8) by showing that increasing the factor H: C1q molar ratio in serum diminishes C4b fixation.

We extended our study to *E. coli* TG1 cells and showed that C1q (Fig. 4 and 5C), the recombinant forms of the globular head modules of human C1q (Fig. 4 and 6D) and factor H (Fig. 4 and 5D) bind specifically to *E. coli*. In addition, factor H was able partially to inhibit C1q binding to *E. coli* cells (Fig. 7A) and *vice versa* (Fig. 7B). When comparing the extent of inhibition by factor H of C1q binding to purified lipid A (Fig. 3A and 3B) and whole *E. coli* cells (Fig. 7A), it is clear that factor H competed out C1q binding more effectively using purified lipid A compared with whole bacterial cells. These results suggest that, on *E. coli*, C1q and factor H share overlapping binding sites, but that C1q and factor H have other distinct, unshared binding sites. C1q or the C1 complex has been reported to bind to several candidate molecules on Gram-negative bacteria. These include porins or outer membrane proteins from *Aeromonas*, *Klebsiella*, *Salmonella* and *Brucella* species (Latsch et al., 1992; Albertí et al., 1996; Eisenschenk et al., 1999; Merino et al., 2005) as well as lipid A and LPS components such as polyribosyl-ribitolphosphate from *Haemophilus influenzae* (Bunse and Heinz, 1993). Other reports indicate other modes of C1q binding via its collagen region, in a manner which does not activate the classical pathway. This has been reported for a 51/57 kDa protein from *E. coli* (van den Berg et al., 1996) and also the LPS component 2-keto-3-deoxyoctonic acid (KDO) from *E. coli* (Zohair et al., 1989). For the interaction of factor H with Gram-negative bacteria, there are many reports of specific factor H-binding bacterial molecules (Kraiczy and Würzner, 2006; Zipfel et al., 2007). Factor H binding to *Neisseria gonorrhoeae* and *N. meningitidis* has been characterized. Factor H is reported to bind to sialylated lipooligosaccharides (LOS) in gonococci, but not in meningococci (Gulati et al., 2005; Schneider et al., 2006). The protein por1A of gonococci and a 33 kDA protein in meningococci bind factor H, as does the protein YadA of *Yersinia* (Kraiczy and Würzner, 2006; Gulati et al., 2005; Schneider et al., 2006). There are no previous reports of direct binding of isolated factor H to *E. coli*,

although binding of factor H during complement activation in serum has been observed (Kubens et al., 1989; Maruvada et al., 2008). These reports do not distinguish direct binding from binding via surface C3b.

The deposition of C4b on lipid A-coated wells by serum depleted of factor H and C1q and repleted with various ratios of both proteins was also examined (Fig. 8). With an increase in the molar ratio of factor H to C1q within the physiological ratio (about 5:1 up to 30:1), there was a steady decrease in the amount of C4b deposited on the lipid A coated wells. Similarly, C4b deposition increased when the ratio of factor H to C1q was decreased below the physiological ratio. These results are consistent with C1q (in the form of C1) and factor H in serum competing for binding to lipid A, resulting in either an increase or decrease in the activation of the classical pathway. Under physiological conditions, factor H may serve as a downregulator of bacterially-driven inflammatory responses, thereby finetuning and balancing the inflammatory response in infections with Gram-negative bacteria.

Serum amyloid P component (SAP), which is constitutively expressed in plasma at a level of about 30–50 μg/mL, has been shown to prevent LPS-mediated activation of the classical pathway (de Haas et al., 2000). SAP binding to the lipid A component of LPS prevented the deposition of C1q and hence interfered with the antibody-independent activation of the classical pathway. SAP binding, however, did not interfere with antibody-mediated C1q activation by LPS/antibody complexes nor alternative pathway activation. In contrast, C1q has been suggested to bind SAP and activate the classical pathway (Ying et al., 1993), but SAP bound to lipid A might present itself in a conformation which is unsuitable for C1q binding and/or classical pathway activation. The inhibition of C1q binding to Gram-negative bacteria by SAP, and in our study, by factor H, might therefore influence the pathophysiology of infection with such bacteria. It is also likely, although this is outside the scope of this study, that factor H may modulate lipid A-mediated macrophage activation that is enhanced by C1q. Thus, factor H may have a scavenging role as an anti-inflammatory mediator where the inflammatory reactions involve interaction between C1q and bacteria or LPS.

MATERIALS AND METHODS

Buffers

The following buffers were used: Veronal buffers (VB, 5 mmol/L sodium barbital, 142 mmol/L NaCl, pH 7.4; VB^{2+}, 5 mmol/L sodium barbital, 142 mmol/L NaCl, 0.15 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, pH 7.4) and SDS-PAGE sample buffer (0.2 mol/L Tris-HCl, 8 mol/L urea, 2% *w/v* SDS, pH 8.0).

Purified proteins and antibodies

C1q was purified from pooled human serum using affinity chromatography on IgG-Sepharose (Tan et al., 2010). Human factor H, purified by monoclonal antibody affinity chromatography (Sim et al., 1993) was supplied by BE Moffatt, MRC Immunochemistry Unit, Oxford. The globular head regions of human C1q 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., 2003).

Radioiodination of proteins

Factor H was radioiodinated with ^{125}I as previously described (Tan et al., 2010). Typical specific activity obtained was 1.1×10^7 cpm/μg (counting efficiency of 70%). C1q, ghA, ghB and ghC were radioiodinated by a less oxidizing procedure (Tan et al., 2010). Typical specific activities were $(2.8–9.2) \times 10^5$ cpm/μg for C1q and $(1.5–1.9) \times 10^6$ cpm/μg for ghA, ghB and ghC. For some experiments, radioiodinated components were diluted with unlabelled material to reduce the specific activity.

Preparation of normal human serum and serum depleted of both C1q and factor H

Plasma pooled from at least 20 donors (HD Supplies, Aylesbury, UK) was recalcified to obtain serum by adding 1 mol/L $CaCl_2$ to a final concentration of 16 mmol/L and the plasma was left to clot overnight at 4°C. The serum was then collected by filtering through muslin. Serum depleted of C1q and factor H was made as described by Tan et al. (2010).

Depletion of C1q was tested by hemolytic assay as previously described (Tan et al., 2010). To measure the extent of factor H depletion, the activity of factor H as a cofactor for factor I-mediated cleavage of C3b/inactive C3 was tested. ^{125}I-labelled-C3 (NH_3) was used as the substrate (Sim and Sim, 1983). This was supplied by Dr. Stefanos Tsiftsoglou, MRC Immunochemistry Unit, Oxford. Serial 2-fold dilutions of control and depleted sera were diluted with PBS-EDTA containing 5 μg/mL soybean trypsin inhibitor (Type IS-SBTI: Sigma) and incubated with a fixed amount of ^{125}I-C3(NH_3) for 12 h at 37°C. The reaction was stopped with SDS-PAGE sample buffer containing 20 mmol/L DTT. The samples were run on a 10% SDS-PAGE gel (Laemmli, 1970), dried down and exposed to X-ray film (Fuji RX, Fuji Photo Film UK Ltd., London, UK) in autoradiography cassettes with intensifying screen for 8 h at −70°C. The extent of C3(NH_3) cleavage by factor I in serum is proportional to the amount of its co-factor, factor H, present in serum. The amount of cleavage was judged by the loss of the C3(NH_3) α chain visualized on the X-ray film. The results showed that factor H was depleted by 75% relative to the control serum.

Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, B, C to solid-phase lipid A

Diphosphoryl lipid A from *E. coli* (Sigma L-5399) was used throughout. Lipid A (5 μg/well) in methanol:chloroform (2:1 *v/v*) solution was added to Maxisorp microtitre plate wells (Nunc, Kamstrup, Denmark) and solvents were allowed to evaporate at 4°C overnight. The wells were then blocked with 10 mmol/L potassium phosphate buffer pH 7.0 + 500 μg/mL ovalbumin for 2 h at room temperature. ^{125}I-C1q, ^{125}I-factor H and ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC were separately added to lipid A coated wells (10,000 cpm/well of ^{125}I-C1q or ^{125}I-factor H, 150,000 cpm/well of ^{125}I-ghA,

^{125}I-ghB and ^{125}I-ghC) and incubated for 1 h at room temperature in a final volume of 100 µL of VB^{2+}. The wells were then washed three times with 200 µL of VB^{2+}. Two hundred microliters of 0.1 mol/L NaOH was added to each well and allowed to incubate for 5 min to dissociate bound proteins and 180 µL of the supernatant was collected and counted in a γ-counter.

Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to lipid A liposomes

Multilamellar lipid A liposomes were prepared according to established methods (New, 1990). The liposome preparations were composed of 25 molar percent dipalmitoylphosphatidylcholine (PC), 45 molar percent cholesterol (CHOL) with 30 molar percent of lipid A. Control PC liposomes contained only 55 molar percent PC and 45 molar percent CHOL. Quantitative determination of total phospholipids was performed by using the Stewart assay (Stewart, 1980). One hundred microliters of multilamellar phospholipid vesicles (100 µg/mL) were mixed with 200 µL of VB^{2+}+ 750 µg/mL ovalbumin containing either 90,000 cpm ^{125}I-C1q, or 100,000 cpm ^{125}I-factor H or 150,000 cpm ^{125}I-ghA or ^{125}I-ghB or ^{125}I-ghC in 1.7 mL conical ultracentrifuge tubes which had been blocked overnight with 500 µL of VB^{2+}+ 1 mg/mL ovalbumin and incubated for 1 h at room temperature. The tubes were then spun at 10,000 *g* in a microfuge for 10 min and the supernatant was removed. The pellet was washed once with 300 µL VB^{2+}, spun and the pellet was counted in a γ-counter.

Saturation binding of ^{125}I-C1q to lipid A-coated wells and liposomes

A fixed amount of ^{125}I-C1q (250 ng/well or reaction) was premixed with various quantities of unlabelled C1q (0–3.75 µg) to lower the specific activity, so that saturation could be achieved without using high levels of radioactivity. The tubes were then incubated on ice for 1 h after which they were incubated with lipid A coated wells or lipid A liposomes, as described above, and the amount of C1q binding was calculated from the radioactivity bound and the known specific activity.

Inhibition of ^{125}I-C1q binding to lipid A-coated wells and lipid A liposomes by ghA, ghB, ghC or factor H

A fixed amount of ^{125}I-C1q in VB^{2+} (3 µg of protein/well or reaction) was incubated with various quantities of unlabelled ghA, ghB, ghC or factor H (0–54 µg for factor H, 0–18 µg for ghA, ghB, ghC) for 1 h on ice. The mixtures were then placed on lipid A-coated wells, or in contact with lipid A liposomes in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+} + 1 mg/mL ovalbumin) containing 100 µL of lipid A liposomes (100 µg/mL) for 1 h at room temperature. The lipid A coated wells or lipids A liposomes were washed and the amount of ^{125}I-C1q bound was determined as above.

Binding of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC to *E. coli*

E. coli TG1 was grown in Luria-Bertani broth (50 mL) at 37°C with vigorous agitation on a shaker to a concentration of 1 × 10^9 cells/mL. The bacterial cells were harvested by centrifuging at 5000 *g* for 10 min at room temperature. The pellet was washed twice and resuspended in VB^{2+} + 0.5 mg/mL ovalbumin. One hundred microliters of VB^{2+} + 0.5 mg/mL ovalbumin containing 125 ng of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC was incubated with 200 µL of *E. coli* (1 × 10^9 cells/mL in VB^{2+}-0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+} + 1 mg/mL ovalbumin) at 37°C for 1 h with mixing every 15 min. Triplicates of the reaction mix (50 µL aliquots) were layered on 200 µL of 15% sucrose (d = 1.05 g/mL) in 0.4 mL microcentrifuge tubes. The tubes were spun for 1 min at 10,000 *g* and the tubes were snapfrozen in liquid nitrogen. The tips were cut off and the amount of radioactivity bound to the bacterial pellet was counted. A correction for non-specific entrapment of ^{125}I-C1q, ^{125}I-factor H, ^{125}I-ghA, ^{125}I-ghB and ^{125}I-ghC was done by taking an aliquot of the cell mix at the start of the experiment (within 30 s of mixing), spinning it down and quantifying the amount bound as described above.

Saturation binding of ^{125}I-C1q and ^{125}I-factor H to *E. coli*

^{125}I-C1q and ^{125}I-factor H (50 ng/reaction) were mixed with varying quantities of unlabelled C1q and factor H in 100 µL of VB^{2+} + 0.5 mg/mL ovalbumin respectively for 1 h on ice. The mix was then incubated with 200 µL of *E. coli* (1×10^9 cells/mL in VB^{2+}+ 0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+}+ 1 mg/mL ovalbumin) and incubated at 37°C for 1 h with mixing every 15 min. The quantity of ^{125}I-C1q and ^{125}I-factor H bound was quantified as described above.

Specific binding of ^{125}I-C1q and ^{125}I-factor H to *E. coli*

To examine if the binding of ^{125}I-C1q or ^{125}I-factor H to *E. coli* was specific, 0.55 µg of ^{125}I-C1q or 0.80 µg of ^{125}I-factor H was incubated with increasing quantities of unlabelled C1q (0–3 µg) or factor H (0–10 µg), respectively, in 100 µL of VB^{2+} + 0.5 mg/mL ovalbumin and incubated on ice for 1 h. The mix was added to 200 µL of *E. coli* (1×10^9 cells/mL in VB^{2+} + 0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+}+ 1 mg/mL ovalbumin) and incubated at 37°C for 1 h with mixing every 15 min. The quantity of ^{125}I-C1q or ^{125}I-factor H bound was quantified as above.

Saturation binding of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to *E. coli*

^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC (50 ng/reaction) was mixed with varying quantities of corresponding unlabelled recombinant globular head proteins in 100 µL of VB^{2+} + 0.5 mg/mL ovalbumin respectively for 1 h on ice. The mix was then incubated with 200 µL of *E. coli* (1 × 10^9 cells/mL in VB^{2+} + 0.5 mg/mL ovalbumin) at 37°C for 1 h with mixing every 15 min. The quantity of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC bound was quantified as above.

Specific binding of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to *E. coli*

To examine if the binding of ^{125}I-ghA, ^{125}I-ghB or ^{125}I-ghC to *E. coli* was specific, 1.55 µg of ^{125}I-ghA, 2.05 µg of ^{125}I-ghB or 3.05 µg of ^{125}I-ghC was incubated with increasing quantities of the corresponding unlabelled ghA, ghB and ghC in 100 µL of VB^{2+}0.5 mg/mL ovalbumin and incubated on ice for 1 h. The mix was added to 200 µL

of *E. coli* (1×10^9 cells/mL in VB^{2+} + 0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+}-1 mg/mL ovalbumin) and incubated at 37°C for 1 h with mixing every 15 min. The radioactivity bound was quantified as above.

Cross-inhibition of ^{125}I-C1q and ^{125}I-factor H binding to *E. coli* by unlabelled factor H and C1q, respectively

^{125}I-C1q (0.55 µg) or ^{125}I-factor H (0.80 µg) was incubated with increasing quantities of unlabelled factor H or C1q respectively for 1 h on ice in 100 µL of VB^{2+} + 0.5 mg/mL ovalbumin. The mix was added to 200 µL of *E. coli* (1×10^9 cells/mL in VB^{2+} + 0.5 mg/mL ovalbumin) in 1.7 mL conical ultracentrifuge tubes (blocked overnight with 500 µL of VB^{2+} + 1 mg/mL ovalbumin) and incubated at 37°C for 1 h with mixing every 15 min. The quantity of ^{125}I-C1q or ^{125}I-factor H bound was quantified as above.

Assessment of C4b deposition, on lipid A coated-wells, from serum containing different C1q to factor H ratios

Serum depleted of C1q and factor H was diluted with 1 volume of VB^{2+} and repleted at various molar ratios of factor H to a fixed amount of C1q by adding back the purified proteins in VB^{2+}. The various serum samples were incubated on ice for 1 h before incubation on lipid A coated wells (blocked for 2 h with VB^{2+} + 1 mg/mL ovalbumin) for 1 h at 37°C. The wells were then washed thrice with VB^{2+} before adding to the wells 100 µL of 1 in 500 dilution of affinity purified chicken polyclonal anti-human C4-alkaline phosphatase conjugate (Immune Systems Limited, Devon, UK) in VB^{2+} and incubating at room temperature for 1 h. Following further washes with 200 µL of VB^{2+}, color was developed at 37°C with 100 µL buffered p-nitrophenyl phosphate (pNPP) substrate (Sigma, N2770). The absorbances were read at 405 nm. The positive control used was control serum that was not depleted of C1q or factor H but was diluted to the same extent. The negative control was the C1q and factor H depleted serum with no added C1q or factor H.

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ABBREVIATIONS

LPS, lipopolysaccharide; ghA, ghB and ghC, the carboxy-terminal region of human C1q A, B and C chains, respectively; VB^{2+}, Veronal Buffer; $DGVB^{2+}$, Dextrose Gelatin Veronal Buffer; MBP, maltose binding protein; CHOL, cholesterol; CL, cardiolipin; PC, dipalmitoylphosphatidylcholine; gC1q, globular domain of C1q; OVA, chicken ovalbumin

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