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C1q and C4b Bind Simultaneously to CR1 and Additively Support Erythrocyte Adhesion

Sander W. Tas,* Lloyd B. Klickstein,† Sergei F. Barbashov,* and Anne Nicholson-Weller2*

Previously, we showed that soluble C1q bound specifically to CR1 on transfected cells. If the CR1-C1q interaction were to participate in immune complex clearance, then this interaction should support E adhesion. Using a tip plate adhesion assay, we found that immobilized C1q mediated adhesion of human E. E binding to C1q was specifically inhibited by polyclonal anti-CR1 Fab fragments. Intact C1 was not efficient as an adherence ligand until it was treated with EDTA or the C1 inhibitor to remove the C1r2C1s complex from C1, leaving C1q. Titration of C1q alone, C4b alone, and C1q + C4b indicated that the two complement ligands were additive in their ability to support CR1-mediated adhesion of E. Analysis of binding to immobilized CR1 using a BIAcore instrument documented that C1q, C4b, and C3b binding were independent events. Additionally, C1q-dependent binding of immune complexes and heat-aggregated IgG to E was documented. These experiments confirm that the immune adherence receptor in humans, CR1, is the single receptor for all of the opsonic ligands of complement, provide evidence for a single C1q binding site on LHR-D of CR1, and suggest that C1q may participate in immune clearance. The Journal of Immunology, 1999, 163: 5056–5063.

Complement receptor type 1 (CR1, CD35) is an integral membrane glycoprotein expressed on E, neutrophils (PMN),3 eosinophils, mononuclear phagocytes, follicular dendritic cells, B lymphocytes, a subset of T lymphocytes, glomerular podocytes (reviewed in Ref. 1), and hypoxic endothelial cells (2). The extracellular domain of CR1 is comprised of repeats, called short consensus repeats (SCR), or complement control protein repeats. The SCR of CR1 are further organized into four long homologous repeats, designated LHR-A, LHR-B, LHR-C, and LHR-D, in the most common CR1 allotype (3). LHR-A is the preferred binding site for C4b, and LHR-B and LHR-C are two tandem C3b binding sites (4–6). LHR-D was proposed as a binding site for C1q, but it was not shown to be the only binding site for C1q (7).

The major role of CR1 on E is to mediate the binding (8–11) and transport to the liver and spleen (12–14) of immune complexes (IC) or particles that have fixed complement. The major role of CR1 on phagocytic cells is to mediate the adherence and phagocytosis of complement-opsonized pathogens (15).

Although many in vitro functions of C1q have been described, the only clear phenotype in humans and mice deficient in C1q is autoimmune disease. Deficiency of C1q is associated with about a 90% chance of acquiring a lupus-like syndrome during childhood (reviewed in Ref. 16). The risk of autoimmune disease associated with C4 deficiency is not known precisely, but it is close to the risk of C1q deficiency, whereas the risk associated with C2 deficiency falls to 30%. Deficiency of C3 and the later complement components is not associated with any significant risk of autoimmune disease (reviewed in Ref. 17). Mice deficient in C1q develop autoantibodies similar to those found in human lupus erythematosus (SLE) as well as glomerulonephritis (18). The mechanism by which deficiency of C1q causes autoimmune disease is unclear; however, there is experimental evidence for two functional roles of C1q, which might be relevant to autoimmunity. First, C1q recognizes and binds to apoptotic cells (19), and may participate in the clearance of autoantigens. Second, C1q binds to the E immune adherence receptor, CR1 (7), which suggested that C1q may participate directly in clearance of IC. If C1q were to participate in clearance of IC by mediating binding to CR1, we would expect the CR1-C1q interaction to support E adhesion, as do the CR1-C4b and CR1-C3b interactions.

In this study, we extend our previous findings by examining the binding of C1q to CR1 expressed on E. Immobilized C1q supported firm E adhesion in a CR1-dependent fashion using the tip plate adhesion assay. These assays and BIAcore analysis of the interaction of purified proteins confirmed that C1q and C4b binding to CR1 were independent events. C1q and (C3b)2 also bound independently to CR1, despite the fact that the C3b binding sites in LHR-B and LHR-C are adjacent to the C1q binding site in LHR-D. Thus, C1q is capable of participating in clearance of intravascular IC. The finding that C1q, (C3b)2, and C4b bind independently to CR1 suggests the presence of a single C1q binding site on CR1.

Materials and Methods

Reagents

The following reagents were purchased as noted: gelatin (porcine skin), hen OVA, CaCl2, MgCl2, D-glucose (Sigma, St. Louis, MO), Tris (ICN...
Isolation and purification of human E

Three milliliters of venous blood were routinely drawn from a healthy volunteer, whose E expressed high levels of CR1 (20), and collected into a glass tube containing 0.098 ml 7.5% EDTA solution (K3) (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The blood was centrifuged for 4 min at 1200 × g after centrifugation, the plasma and buffy coat layers were removed and E were washed twice with 5 ml adhesion buffer. E were centrifuged again for 4 min at 1200 × g and suspended in 5 ml adhesion buffer. E were quantified using a hemacytometer and suspended in adhesion buffer at 5 × 10^7 cells/ml.

Human proteins

C1q was isolated from human serum by fractional elugobulin precipitation, as described (7). Analysis of C1q purity was assessed by SDS-PAGE (12% gel; Novex, San Diego, CA). C1q stalks were made by pepsin digestion of intact C1q (21). In brief, C1q in sodium acetate buffer (100 mM sodium acetate, 150 mM NaCl, pH 4.4) was incubated with pepsin at a C1q:pepsin ratio of 30:1 for 3.5 h at 37°C. The reaction was stopped by addition of 10% PBS, and the mixture was passed over a gel filtration column (TSK G4000SWXL; Supelco, Bellefonte, PA) equilibrated in 2× PBS. The peak containing the collagen stalks was collected.

(C3b)2, was made as described (7). C3 purified from fresh human plasma (22) was treated with trypsin to produce C3b (23). The trypsin was inactivated by addition of disopropylfluorophosphate, and the C3b was purified by gel filtration (Sephacryl S300; Pharmacia LKB Biotechnology, Piscataway, NJ). The fractions containing C3b were pooled, aliquoted, and stored at −80°C.

Macromolecular C1, C1-esterase inhibitor (C1-INH), C4, and C4b were purchased from Advanced Research Technologies (San Diego, CA). Soluble human rCR1 (rsCR1) was provided by Avant Immunotherapeutics (Needham, MA). C-reactive protein that was isolated from human pleural fluid was provided by Alok Agrawal (University of Alabama, Birmingham).

Anti-CRI Fab fragments

Ammonium sulfate precipitation and protein A affinity chromatography were used to prepare an IgG fraction of rabbit anti-human CR1 (25). The anti-CRI IgG (20 mg) was digested to Fab fragments using the ImmunoPure Fab Preparation Kit (Pierce, Rockford, IL), which is based on immobilized papain. The digest was subsequently passed over a protein A column (Pierce), and the nonretained fraction showed a 50-kDa band when analyzed by SDS-PAGE with Coomasie staining and a 25-kDa band under reducing conditions. All procedures followed the manufacturer’s protocol, provided in the kit. An aliquot of the polyclonal Fab anti-CRI preparation was passed through an affinity column of rsCR1 immobilized on acrylamide beads (3 M Emphaze; Pierce). Absorbed Fab were obtained from the drop-through fraction. All fractions of Fab were first extensively dialyzed against PBS, and the protein concentrations were determined by the micro BCA (bicinchoninic acid) method (Pierce), using BSA as a standard.

Surface plasmon resonance analysis of binding was performed using a BIAcore instrument (Pharmacia). This technique measures in real time the association and dissociation of unlabeled ligand to an immobilized receptor, or vice versa, by changes in the adjacent refractive index (27). rsCR1 (800 μg/ml in 10 mM citrate, pH 4.8 buffer) was immobilized to a CM5 sensor chip (Pharmacia) by carbodiimide (EDC) and N-hydroxysuccinimide (NHS), according to the manufacturer’s instructions (28). Binding studies were performed in PBS at 25°C using a flow rate of 5 ml/min. For analysis of C1q and C4b binding, the immobilized rsCR1 was regenerated for subsequent analysis by washing with 0.5 M NaCl. In (C3b)2-binding studies, the CR1 was regenerated by washing with 0.1 M sodium citrate (pH 4.8). Data were analyzed using BIAcore software (BIA Evaluation; Pharmacia).

The binding of heat-aggregated IgG (HAG) and IC to human E

HAG was made by incubating protein G-isolated IgG at 63°C for 30 min, as described (29). Subsequently, the mixture was centrifuged and the supernatant was applied to a TSK G4000SWXL (Supelco) gel filtration column equilibrated in 0.4 M NaCl, phosphate buffer, pH 7.4, and the major peak at m.w. 800,000–2,000,000 was collected. IgG for IC was made by activation of human immunoglobulin G (anti-129I) using carbodiimide (Bayer, Elkhart, IN) using agarose-cyanogen bromide-coupled tetanus toxoid (BayTet; Wyeth-Lederle Labs, Marietta, PA). The affinity-purified IgG fraction (45 μg) was incubated with tetanus toxoid (10 μg) in 210 μl PBS for 1 h at 4°C, and subsequently the reaction mixture was made 0.4 M with NaCl and centrifuged. The supernatant was applied to a TSK G4000SWXL (Supelco) gel filtration column, as described above, and the major protein peak at m.w. 800,000–2,000,000 was collected. Both the HAG and the IC were separately radiiodinated with sodium iodide, 129I (New England Nuclear, Boston, MA), using Iodogen (Pierce) to sp. act. of 95 × 10^6 cpm/μg and 33 × 10^6 cpm/μg, respectively.

The final binding mixture (340 μl total, E-binding buffer) contained C1q (25 μg/ml), human E (25 × 10^6/ml), and 100 ng/ml of either 129I-labeled HAG or 129I-labeled IC. Incubation was conducted for 40 min at room temperature with intermittent agitation, and subsequently three replicate 100-μl samples were removed, separately layered on 300 μl of oil (85% dibutyl, 15% dinonyl phthalate), and centrifuged for 1 min at
10,000 × g. The tips of tubes containing the E pellet were cut off and counted in a gamma counter.

Results

Measurement of E adhesion to immobilized complement ligands

As positive controls for the tip plate E adhesion assay, the well-defined ligands for CR1, namely (C3b)2 and C4b, were studied. In this adhesion assay, purified complement fragments were immobilized on plastic dishes and E was allowed to bind. After washing, specific binding of E to complement fragments (Fig. 1A, upper panel) was evident compared with blocked areas of the plate (Fig. 1A, lower panel). A plot of the input protein concentration vs the number of adherent E/mm² indicated a dose-dependent increase of E adhesion to the immobilized C4b and (C3b)2 dimers (Fig. 1B).

Maximal adhesion was seen at C4b and (C3b)2 concentrations of 5 μg/ml and yielded 19,046 ± 306 and 17,993 ± 310 cells/mm², respectively. In contrast, under the equivalent conditions, 120 ± 11.5 cells/mm² were adherent to adjacent gelatin-blocked areas of plastic. The efficient binding of E to C4b and C3b dimers indicated that the adhesion assay conditions were appropriate. The adhesion assay was repeated with plates coated with increasing amounts of C1q, and a dose-dependent increase in adherent E was observed (Fig. 1C). C1q-mediated adhesion was saturable, and maximal adhesion was seen at an input C1q concentration of 15 μg/ml. These data confirm that E are capable of adherence to C1q, as well as C4b and (C3b)2.

Adhesion of E to C1q was blocked by polyclonal anti-CR1 Fab

To show that CR1 was the membrane receptor involved in the binding of E to the immobilized ligands, E were preincubated with polyclonal anti-CR1 Fab at a concentration of 100 μg/ml before being added to the petri dishes. As a control for the Fab treatment, a duplicate sample of E was preincubated with Fab anti-CR1 that had been absorbed with immobilized rsCR1. All anti-CR1 reactivity was removed with this absorption, as assessed by ELISA (see Materials and Methods). Anti-CR1 Fab (100 μg/ml) completely blocked the adhesion of the E to C4b and (C3b)2 immobilized on the plates, whereas anti-CR1 Fab (100 μg/ml) absorbed with immobilized rsCR1 had no effect on E binding to these immobilized complement fragments (Fig. 2A). To assess whether E also bind to C1q through CR1, C1q (10 μg/ml) was immobilized in marked areas, as described above, and E were preincubated with different concentrations of the anti-CR1 Fab. Anti-CR1 Fab at 25, 50, or 100 μg/ml resulted in 17.4%, 96.4%, and 100% inhibition of the binding to immobilized C1q, respectively (Fig. 2B). The control Fab (100 μg/ml) that had been absorbed with immobilized rsCR1 had a minimal effect. These data indicate that E bind to C1q, as well as C4b and (C3b)2 through CR1.
Binding of E to the collagen region of C1q through CR1

There are two domains of C1q: a collagen domain, which is obtained from a pepsin digest of C1q (21); and a globular domain, which is obtained from a collagenase digest of C1q (30). The collagen domain of C1q bound to immobilized CR1 (7). To demonstrate that the collagen stalks of C1q also mediate adhesion, different amounts of collagen stalks were coated on petri dishes, as described above. The binding of E to C1q stalks was dose dependent, and this binding could be blocked completely by anti-CR1 Fab (100 μg/ml) (Fig. 3). These data confirm that the collagen domain of C1q can mediate C1q binding to erythrocyte CR1.

Adhesion of E to C1 treated with C1 inhibitor or EDTA

Macromolecular C1 consists of 1 molecule C1q, complexed to two molecules of each C1r and C1s (C1r2C1s2) (31). It is known that intact C1 binds poorly to cells, whereas C1q alone binds well (32).

We compared the adhesion of E to C1 before and after the removal of the C1r2C1s2 complex. The C1r2C1s2 complex was removed by chelation of Ca2+ using 5 mM EDTA, or by complexing C1rC1s with C1-INH, which binds stoichiometrically and irreversibly with C1rC1s (reviewed in Ref. 33).

C1 (10 μg/ml) was either incubated alone, or incubated with C1-INH (20 μg/ml, 7.2-fold molar excess of C1-INH over C1r2C1s2 (34)) or 5 mM EDTA in adhesion buffer and coated to marked areas on petri dishes. Addition of C1-INH or EDTA resulted in a 7.6-fold and 7.9-fold increase, respectively, in the number of E bound to the coated areas compared with C1 before the removal of C1r2C1s2 (Fig. 4). These data are consistent with...
C1r, C1s, C2, and C4b, interfering with C1q-CR1 binding, either by steric hindrance or direct competition with CR1 for an SCR binding site on C1q.

**Kinetic measurement of \((C3b)_2, C4b, \text{ and } C1q\) binding to CR1**

Although C4b, \((C3b)_2\), and C1q bind to different domains on rsCR1 (LHR-A, LHR-B or LHR-C, and LHR-D, respectively), an important issue is whether the binding of one ligand might influence that of another. Surface plasmon resonance analysis of binding was performed using a BIAcore instrument. This technique measures in real time the association and dissociation of unlabeled ligand to an immobilized receptor, or vice versa, by changes in the adjacent refractive index (27). rsCR1 was covalently coupled to a CM5 dextran chip and resulted in the net addition of 10,851 resonance units (RU) to the chip.

The reported apparent \(K_D\) for \((C3b)_2\) binding to CR1 have ranged from 4.5 to 30 nM (6, 7, 35–38). Most recent findings support a \(K_D\) between 18 and 30 nM (6, 7, 38). The apparent \(K_D\) for C1q binding to CR1 calculated from kinetic data was 3.9 nM (7). These reports were used to select saturating concentrations of \((C3b)_2\) and C1q, 66.67 and 100 nM, respectively, which were employed to fully occupy available binding sites on rsCR1. C4b was used at 1.5 \(\mu\)M, the highest available concentration, which is greater than the reported \(K_D\) for C4b binding to CR1 (39), but not saturating. C4b at 1.5 \(\mu\)M yielded a maximal signal of 440 RU, and C1q at 0.1 \(\mu\)M resulted in a maximal signal of 249 RU, when analyzed in PBS (Fig. 5A). A mixture of the two proteins at the same concentrations as used individually resulted in a maximal signal of 677 RU, close to the sum of the RU observed when each protein was analyzed separately. Similarly, \((C3b)_2\) at a concentration of 66.67 nM resulted in a maximal signal of 777 RU, and reanalysis of C1q at 0.1 \(\mu\)M yielded a maximal signal of 229 RU. A mixture of the two proteins at the same concentrations as used individually resulted in a maximal signal of 1006 RU, which was precisely additive (Fig. 5B). These data, together with our previous finding of a C1q binding site in the region of CR1 located within LHR-D and/or SCR 29 and 30, support the presence of a single C1q binding site on CR1 that does not overlap with the sites for C3b and C4b.

**Additive binding of C1q and C4b to CR1 on E**

The BIAcore data demonstrated that the binding of C4b and C1q to rsCR1 was independent, which suggested that C1q and C4b should be at least additive in their ability to support adhesion of CR1-bearing cells. We immobilized C1q and C4b (at various input concentrations) alone or together, on petri dishes, and compared the E adhesion with the adherence of E to the dimeric ligand, \((C3b)_2\). The data are plotted in individual adhesion curves of \((C3b)_2\), C1q, C4b, and an adhesion curve for coimmobilized C1q and C4b (Fig. 6). In this experiment, half-maximal adhesion was observed at input concentrations of 19 and 23 nM for C1q and C4b, respectively, when immobilized separately. Half-maximal adhesion of coimmobilized C1q + C4b, and \((C3b)_2\) was seen at input concentrations of 14 and 10 nM, respectively, approximately half of either protein analyzed separately, consistent with an additive effect. Thus, the independent binding of C1q and C4b to CR1 resulted in augmented E adhesion.

**C1q-dependent binding of HAG and tetanus toxoid IC to E**

The CR1 of primate E has a major role in the clearance of IC, and thus it was relevant to see whether C1q could mediate the adherence of radiolabeled aggregated IgG, either in the form of HAG complexes or IC, to human E. E were mixed in E-binding buffer with a fixed amount of radiolabeled HAG and varying concentrations of C1q (0–42 \(\mu\)g/ml). The cpm associated with the cells was directly dependent on the amount of C1q added, with half-maximal binding at 4.65 \(\mu\)g/ml and saturation at 14 \(\mu\)g/ml (Fig. 7). \(^{125}\text{I}-\) labeled tetanus toxoid/anti-tetanus toxoid IgG IC also demonstrated C1q-dependent binding to E: in the absence of C1q, there were 400 \(\pm\) 35 cpm (\(n = 3\)) associated with the E pellet; in the presence of 25 \(\mu\)g/ml C1q, there were 11,252 \(\pm\) 638 cpm (\(n = 3\)) associated with the E pellet. These experiments clearly indicate that C1q can mediate the adherence of IC to E.

**Discussion**

The receptor for C1q has remained an enigma since the original description of C1q binding to lymphocytes (40). More recently, it has become clear that there are C1q-binding factors on cells that often bind the globular domains of C1q (reviewed in Ref. 41), whereas at least on PMN and monocytes, binding is through the collagen domain of C1q (32, 42). In this study, we show that C1q (Fig. 1B) and specifically the collagen domain of C1q (Fig. 3) bound to CR1 expressed on E. E are ideal cells for assessing complement-mediated adhesion, because they lack other complement receptors and integrins, which may complicate interpretation of adhesion assays. Also E, unlike PMN, cannot readily alter their
with E (15 ml; 5 × 10⁷/ml). The dishes were washed twice with adhesion buffer, fixed in paraformaldehyde (2% in PBS), and quantified by microscopy. Results represent the mean of three representative fields in the same marked coated area. Data are expressed as adhesion curves of E to the coated spots as mean number of adherent E/mm² ± SD of a representative experiment performed in triplicate.

Dose dependence of C1q-mediated binding of 125I-HAG to human E. Varying concentrations of C1q (0–42 µg/ml) were mixed with a fixed amount of 125I-HAG and E (25 × 10⁷/ml), as described in Materials and Methods. After an incubation for 40 min at room temperature, three replicate 100-µl samples were removed, and the cells were centrifuged through oil to separate the cell-bound 125I-HAG from the free 125I-HAG. The tips of tubes containing the E pellet were cut off and counted in a gamma counter. The mean ± SE (n = 3) was plotted.

Interestingly, it was not possible to block E binding using available mAbs directed against CR1 (data not shown). These mAbs included 7G9 (46), 9H3 (47), 3D9 (47), YZ1 (48), 6B1.H12, and 3C6.D11 (49). It is possible that the C1q binding site on human CR1 is relatively nonimmunogenic in mice. Furthermore, because some of these mAb block C3b binding to CR1, this is additional support for distinct binding sites for C1q and C3b on CR1.

FIGURE 6. E show additive binding to C4b and C1q coated together on petri dishes. Various concentrations of (C3b)2, C1q alone, C4b alone, or C1q and C4b together at a 1:1 molecular ratio were coated on petri dishes. After washing and blocking, the dishes were incubated for 45 min at RT with E (15 ml; 5 × 10⁷/ml). The dishes were washed twice with adhesion buffer, fixed in paraformaldehyde (2% in PBS), and quantified by microscopy. Results represent the mean of three representative fields in the same marked coated area. Data are expressed as adhesion curves of E to the coated spots as mean number of adherent E/mm² ± SD of a representative experiment performed in triplicate.

FIGURE 7. Dose dependence of C1q-mediated binding of 125I-HAG to human E. Varying concentrations of C1q (0–42 µg/ml) were mixed with a fixed amount of 125I-HAG and E (25 × 10⁷/ml), as described in Materials and Methods. After an incubation for 40 min at room temperature, three replicate 100-µl samples were removed, and the cells were centrifuged through oil to separate the cell-bound 125I-HAG from the free 125I-HAG. The tips of tubes containing the E pellet were cut off and counted in a gamma counter. The mean ± SE (n = 3) was plotted.

FIGURE 8. Schematic model of complement fragment binding sites on CR1. The F(A or CR1*1) allotype is shown. Small circles represent SCR. Black SCR represent the binding sites of the complement fragment specified and aligned above. The preferred ligand is bold; the alternative ligand is in parentheses. The sites for C4b and C3b are as reported (4–6, 50). The general location of the binding site for C1q is denoted by the grey circles (7), but the specific SCR involved in the binding have not been defined.
does not suffice: C4 must be cleaved to prevent autoimmunity. Furthermore, the activation of C4 must occur in a regulated manner, because the unregulated C4 cleavage and resultant relative C4 deficiency that is associated with hereditary angioedema are also associated with a risk of autoimmune disease (54, 55).

C4A greatly augmented IC binding to CR1, compared with the C4B null phenotype is associated with SLE, while the C4B null phenotype is not (reviewed in Ref. 56). In comparisons of the functional activity of C4A compared with C4B, C4A generates more C4b binding to IC (57). When comparable amounts of C4b derived from either C4A or C4B are deposited on IC, the C4b derived from C4A greatly augmented IC binding to CR1, compared with the C4b derived from C4B (58).

Thus, C4b-CR1 interactions are important in the maintenance of self-tolerance. Our data indicate that C1q can augment C4b-mediated adhesion to CR1, and this might be critical if C4b were limiting. Support for a role of C1q as an adhesive ligand, independent of the role of C1q in activating macromolecular C1 for the cleavage of C4, comes from the evidence that C1q deficiency is a more severe risk factor for autoimmune disease than C4 deficiency (51). The C1q- and C4b-dependent step in preventing autoimmunity might be in proper clearance of autoantigens from apoptotic cells, or processing and removal of IC. Alternatively, C1q and C4b may participate in the maintenance of lymphocyte tolerance to self Ags. These possibilities are not mutually exclusive and should be the subject of future studies.

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