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Complement Receptor Type 1 (CR1, CD35) Is a Receptor for C1q

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Summary

Molecular definition of the cellular receptor for the collagen domain of C1q has been elusive. We now report that C1q binds specifically to human CR1 (CD35), the leukocyte C3b/C4b receptor, and the receptor on erythrocytes for opsonized immune complexes. Biotinylated or radioiodinated C1q (*C1q) bound specifically to transfected K562 cells expressing cell surface CR1 and to immobilized recombinant soluble CR1 (rsCR1). *C1q binding to rsCR1 was completely inhibited by unlabeled C1q and the collagen domain of C1q and was partially inhibited by C3b dimers. Kinetic analysis in physiologic saline of the interaction of unlabeled C1q with immobilized rsCR1 using surface plasmon resonance yielded an apparent equilibrium dissociation constant (K_{eq2}) of 3.9 nM. Thus, CR1 is a cellular C1q receptor that recognizes all three complement opsonins, namely, C1q, C3b, and C4b.

Introduction

Complement C1q is the 462 kDa recognition subunit of multimeric C1, which when bound to a complement activating surface, causes the sequential activation of the enzymatic subunits of C1, C1r, and C1s. C1q avidly binds IgG and IgM in immune complexes and thereby "complements" acquired immunity by recruiting the classical complement pathway. In addition, C1q can directly bind DNA (Uwatoko et al., 1990), complexes of C-reactive protein (Jiang et al., 1991), serum amyloid P (Ying et al., 1993), isolated myelin (Vanguri et al., 1982), and urate crystals (Terkeltaub et al., 1983), as well as some gram-negative (Tenner et al., 1984) and gram-positive bacteria (Baker et al., 1982). By directly binding foreign or abnormally expressed host molecules and activating the classical pathway, C1q functions as part of the innate immune system. Whether activated by the acquired or innate immune system, the catalytic function of C1 is tightly regulated by the C1 inhibitor, which covalently binds to the activated enzymatic subunits of C1, C1r, and C1s and removes them from C1q. C1q, still bound to its immune complex or activating substance via its globular domains, is now free to interact with cells bearing receptors for its collagen-like domain. The collagen-like domain, or collagen tail, is what remains of the C1q molecule after the six globular domains are digested away by pepsin (Reid, 1976). The collagen tail consists of the six "stems" of C1q, which come together in the amino half of the constituent chains to form a single "stalk."

Receptors for aggregated or insolubilized C1q were first recognized on lymphocytes (Dickler et al., 1972). Subsequent studies identified monocytes, some PMN, and B lymphocytes as the predominant C1q binding cell types in the peripheral blood (Tenner et al., 1981a). The molecular definition of the C1q receptor(s) has been difficult, which may be explained in part by different methods used to define different receptors on different cell types. Using C1q affinity, Reid and coworkers identified a 60 kDa protein (Malhotra et al., 1988, 1989) that is highly homologous, if not identical, to calreticulin (Malhotra et al., 1992; Stuart et al., 1996).

Initially using a purification procedure also based on C1q affinity, Ghebrehiwet and coworkers have described different forms of membrane-binding C1q molecules ranging from 1–2 × 10^6 M_r (normal B lymphocytes) (Ghebrehiwet et al., 1982) to 70–80k M_r (Raji cells) (Ghebrehiwet et al., 1984; Ghebrehiwet, 1988). The Raji cell C1qR had a domain with significant homology to α5/β1 and the vitronectin receptor, αv/β3 integrin (Ghebrehiwet et al., 1992). More recently, this group has used antibodies against the 60k M_r C1qR (Ghebrehiwet, 1988; Malhotra and Sim, 1989) and identified calreticulin on surface-labeled endothelial cells (Peerschke et al., 1993) and the T cell line MOLT4 (Chen et al., 1994).

Tenner and coworkers investigated the phagocytosis-enhancing and superoxide-enhancing effects of C1q on monocytes and granulocytes. They used a C1q affinity-purified fraction from U937 cells as an immunogen for making MAbs and then selected several MAbs that blocked C1q-enhanced phagocytosis by monocytes and found that the MAbs reacted with a 126k M_r surface protein (Guan et al., 1991). Using degenerate probes based on amino acid sequence, they have identified a cDNA clone that directs expression of a protein with a predicted amino-terminal C-type lectin domain, five EGF-like repeats, a single transmembrane domain, and a short cytoplasmic tail (Nepomuceno et al., 1997). The 126k M_r protein is expressed on both monocytes and neutrophils (Guan et al., 1991).

Results from our laboratory indicated that the regulation of C1q binding on human PMN was similar to the regulation of complement receptor type 1 (CR1) and type 3 (CR3) expression. Biotinylated C1q ($C1q^{bio}$) binding and CR1 and CR3 expression were simultaneously up-regulated by FMLP, and microtubule stabilization with taxol inhibited the up-regulation of $C1q^{bio}$ binding and CR1 and CR3 expression (Jack et al., 1994). These findings suggested to us that the C1q receptor of PMN

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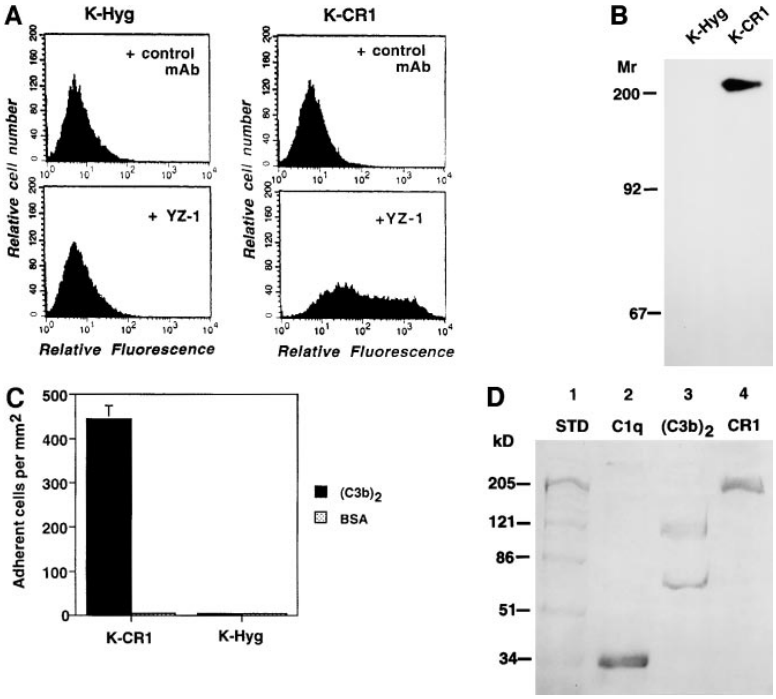

Figure 1. Analysis of CR1-Transfected K562 Cells

(A) FACS analysis of CR1 expression on transfected cells. Samples of cells transfected with the control plasmid pHyg (left panels) or the plasmid containing the cDNA encoding CR1 (right panels) were incubated with class-specific control MAb then FITC-goat anti-mouse IgG (upper panels), or anti-CR1 (YZ-1), followed by FITC-goat anti-mouse IgG (lower panels). The cells were subsequently fixed and analyzed by FACS. The K-Hyg cells (lower left) showed no positive staining for CR1, while the K-CR1 cells (lower right) displayed a broad histogram of positive staining.

(B) Western blot analysis of NP-40 cell lysates of K-Hyg and K-CR1 cells using rabbit anti-CR1 and iodinated protein A as a probe. The K-CR1 cells have a unique 200k M_r band corresponding to intact CR1.

(C) Adherence of transfected K562 cells. K-CR1, but not K-Hyg, bound to C3b immobilized on plastic. Adherent cells were counted by microscopy.

(D) SDS–PAGE analysis under reducing conditions of purified proteins (4 μg/lane) on a 4%–20% slab gel.

either was in the same intracellular storage vesicle as CR1 and CR3 or was identical to CR1 or CR3. The catalytic subunits of C1, C1r, and C1s, which bind C1q (Lepow et al., 1963), have complement short consensus repeat (SCR) motifs (Journet et al., 1986; Leytus et al., 1986; Tosi et al., 1987) and therefore share homology with CR1, which has 30 SCR in its extracellular domain (Klickstein et al., 1987). Thus, it was logical to assess if C1q could bind CR1.

In this paper, we provide evidence, from two different equilibrium binding assays and a kinetic binding assay, that C1q can indeed bind CR1. C1q has at least two binding sites for CR1, presumably involving some of its six identical stems, where C1r and C1s also bind (Siegel et al., 1983). These results indicate that CR1, the receptor normally responsible for binding immune complexes and mediating their transport to the liver and spleen for clearance, can bind to any of the complement fragments that mark immune complexes for clearance, namely C1q, C3b, and C4b.

Results

Characterization of Transfected Cells

The human erythroleukemia cell line K562 was transfected with the plasmid paABCD, which directs the expression of the F allotype of human CR1, together with the plasmid pBSHyg, which directs resistance to the antibiotic hygromycin (K-CR1), or with pBSHyg alone (K-Hyg). The K-CR1 and the control K-Hyg transfectants were characterized for CR1 expression using control MAb and the anti-CR1 MAb YZ-1. There was no YZ-1 staining of the K-Hyg cells, compared with a control MAb (MFC of FITC-second antibody alone = 9.25; MFC of anti-CR1 plus FITC-second antibody = 8.69) (Figure 1A, left panels). In the same analysis, K-CR1 demonstrated a heterogenous pattern of positive staining compared with its control (Figure 1A, right panels). Because the K-CR1 cells were selected for cell surface expression of CR1 by panning but the cells were not cloned, a heterogeneous expression of CR1 was expected. The recombinant CR1 molecule was shown to be intact by immunoprecipitation and Western blot analysis using polyclonal anti-CR1 (Yoon et al., 1985) and iodinated protein A as a probe (Figure 1B). The K-CR1 bound to C3b immobilized on plastic, while K-Hyg did not bind (Figure 1C). SDS–PAGE analysis of the purified proteins used in the experiments confirms their purity and expected molecular structures (Figure 1C).

Measurement of C1q Binding to Transfected Cells

Transfected cells were incubated at 37°C for 30 min with increasing amounts of biotinylated C1q ($C1q^{bio}$) in Ca^{++}- and Mg^{++}-free low ionic strength buffer, HBSS/2. Cells were subsequently incubated with FITC-avidin and analyzed by FACS. A plot of the mean fluorescent channel (MFC) versus $C1q^{bio}$ input indicated specific binding of $C1q^{bio}$ (Figure 2A). The binding was saturable and half maximal binding was seen at a $C1q^{bio}$ concentration of 7.6×10^{-8} M. The C1q binding assay was repeated using ^{125}I-C1q. K-Hyg and K-CR1 cells were incubated with increasing amounts of ^{125}I-C1q for 45 min at room temperature. A dose-dependent increase in ^{125}I-C1q binding was observed (Figure 2B). However, it was not technically possible to reach saturation because the iodinated C1q aggregated at higher concentrations. A 50-fold excess of unlabeled C1q inhibited 82% (mean, n = 3) of 2.6 nM ^{125}I-C1q binding.

C1q Binding to CR1 Immobilized to Microtiter Wells

To circumvent the problem of background C1q binding to other cell surface sites, a recombinant, soluble construct of the entire extracellular domain of CR1 (rsCR1) was immobilized in microtiter wells. Preliminary titration of rsCR1 binding to the plate indicated that 0.5–0.8 μg

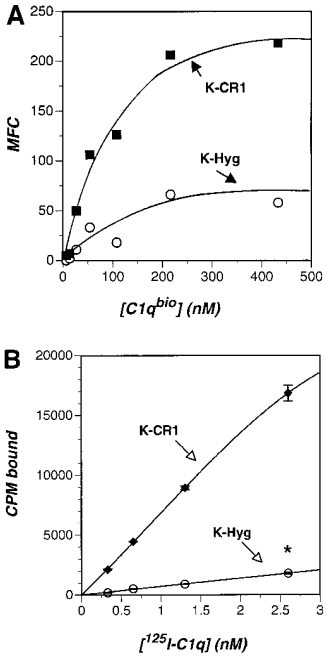

Figure 2. Labeled C1q Binding to Transfected Cells

(A) $C1q^{bio}$ Binding. After blocking nonspecific avidin and biotin binding sites, samples of K-Hyg and K-CR1 (5×10^6 cells) were reacted with increasing concentrations of $C1q^{bio}$ for 30 min at 37°C in low ionic strength buffer (HBSS/2; see Experimental Procedures for details). The cells were subsequently reacted with FITC-avidin, fixed in 1% paraformaldehyde, and analyzed by FACS. The mean fluorescent channel (MFC) of the single dominant peak was determined based on analyzing 20,000 cells. This experiment was repeated two other times with similar net positive binding of $C1q^{bio}$ to K-CR1 cells as compared with K-Hyg.

(B) ^{125}I-C1q binding. Transfected K-Hyg and K-CR1 cells, each at 5×10^6/ml, were incubated with increasing doses of ^{125}I-C1q (specific activity: $1.1 \times 10^6/\mu g$), diluted in low ionic strength buffer (HBSS/2). After a 45 min incubation at room temperature, three replicate 0.1 ml samples were removed from each reaction mixture, layered onto oil, and centrifuged to pellet the cells. The cell pellet containing 5×10^5 cells was cut off and counted. The means of triplicate values ± SE are depicted. At the highest input of ^{125}I-C1q (2.6 nM), a 50-fold excess of unlabeled ligand inhibited specific binding by 82% (*). This experiment was repeated four times with similar results.

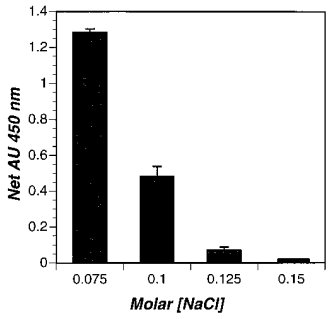

Figure 3. Effect of Ionic Strength on $C1q^{bio}$ Binding to Insolubilized rsCR1

Recombinant sCR1 (5 μg/ml) was used to coat microtiter wells. After washing and blocking, $C1q^{bio}$ (0.5 μg/well), diluted in the respective ionic strength buffer, was added for a 30 min incubation at room temperature. The wells were washed 2× with the respective buffer. Results represent the mean of duplicate values, while the bars depict the range of values. This experiment was repeated with a similar inverse relationship between ionic strength and $C1q^{bio}$ binding.

of rsCR1/well provided optimal C1q binding. C1q binding to cells was facilitated by low ionic strength, but C1q also aggregated at low ionic strength, which can make binding assays technically difficult. To identify an ionic strength closest to normal for the plate assay that would permit reproducible measurement of C1q binding to immobilized rsCR1, we tested the ability of $C1q^{bio}$ to bind under various ionic strength conditions. Adequate binding was measured in 0.1 M NaCl (Figure 3), thus subsequent plate binding studies were done in an equivalent low ionic strength buffer ("0.67 × PBS-Tween" = 67% PBS, 33% dHOH, 0.05% Tween-20, 5.9 mSi at 0°C).

The specific binding of ^{125}I-C1q to immobilized rsCR1 is shown in Figure 4A. Half maximal binding was observed at 0.5 nM of ^{125}I-C1q. The total binding observed in this assay was approximately 30-fold lower than that in the cell binding assay (Figure 2B) because of the higher ionic strength used in the plate assay. Binding of labeled C1q to immobilized rsCR1 was inhibited by native C1q (Figure 4B). A concentration of 0.4 nM unlabeled C1q inhibited ^{125}I-C1q binding by 50%. The collagen "tail" portion of C1q, which was obtained by pepsin digestion of C1q, also competed with ^{125}I-C1q for binding to immobilized rsCR1, which indicated that CR1 binds to the same collagen domain of the C1q molecule as does C1r and C1s (Siegel and Schumaker, 1983). In two experiments, 50% inhibition of ^{125}I-C1q binding was observed with about 10–15 nM of the collagen tails (data not shown). $(C3b)_2$ also inhibited ^{125}I-C1q for binding to CR1, but less efficiently than C1q (Figure 4C). Because $(C3b)_2$ is a well-described ligand for CR1 (Fearon, 1980), this affords additional specificity to the competition assays.

Kinetic Measurement of C3b or C1q Binding to rsCR1

To demonstrate that C1q could bind CR1 under physiologic conditions and to avoid the deleterious effects of labeling procedures on the activity of C1q, 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 (Cullen et al., 1987). In a typical experiment, rsCR1 was covalently coupled to a CM5 dextran chip and resulted in the net addition of 9052 resonance units (RU) to the chip.

To confirm that the coupled rsCR1 was functionally intact and to establish the validity of this method for measuring the binding of complement fragments to CR1, we assessed the ability of a known CR1 ligand, namely soluble $(C3b)_2$, to bind to the coupled rsCR1. Specific binding of $(C3b)_2$ to rsCR1 was observed using five different concentrations of $(C3b)_2$ ranging from 8.3 nM to 67 nM. Figure 5A illustrates the observed association and dissociation of $(C3b)_2$, which was used to derive the association and dissociation rate constants. The association phase of the interaction was modeled as simple bimolecular binding, because the $(C3b)_2$ ligand was employed at a concentration 20- to 60-fold below

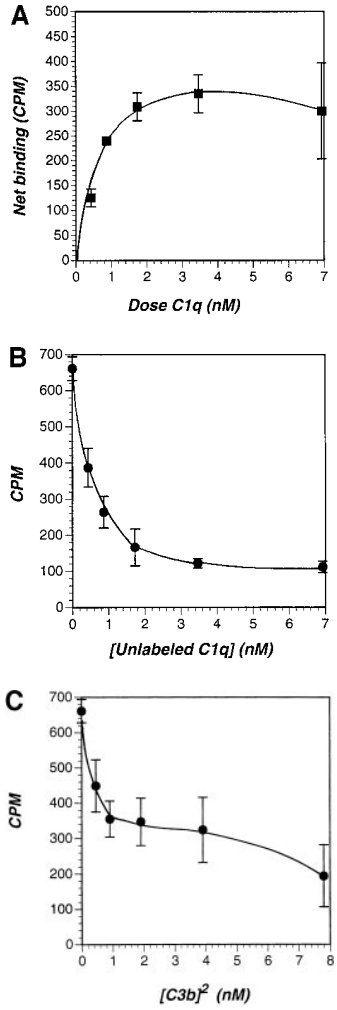

Figure 4. Binding of ^{125}I-C1q to Immobilized rsCR1 and Competition by Unlabeled Ligands

Recombinant sCR1 (8 μg/ml) was immobilized in microtiter wells, and binding was performed in 0.67 × PBS-Tween buffer in the absence of added Ca^{++} and Mg^{++}, as described in the Experimental Procedures.

(A) Direct ^{125}I-C1q binding to plated rsCR1. Results are the means ± SE, n = 4. This experiment was repeated with similar results.

(B) Competition of ^{125}I-C1q binding by native C1q. ^{125}I-C1q (0.87 nM) binding to immobilized rsCR1 was measured in the presence of increasing concentrations of unlabeled C1q in 0.1 M buffer. Means ± SE, n = 3 are plotted. This experiment was repeated with similar results. Unlabeled C1q at 0.4 nM inhibited ^{125}I-C1q binding by 50%.

(C) Competition of ^{125}I-C1q binding by $(C3b)_2$. ^{125}I-C1q (0.87 nM) was added to wells containing immobilized rsCR1 in the presence of increasing amounts of $(C3b)_2$. Results are the means ± SE, n = 4.

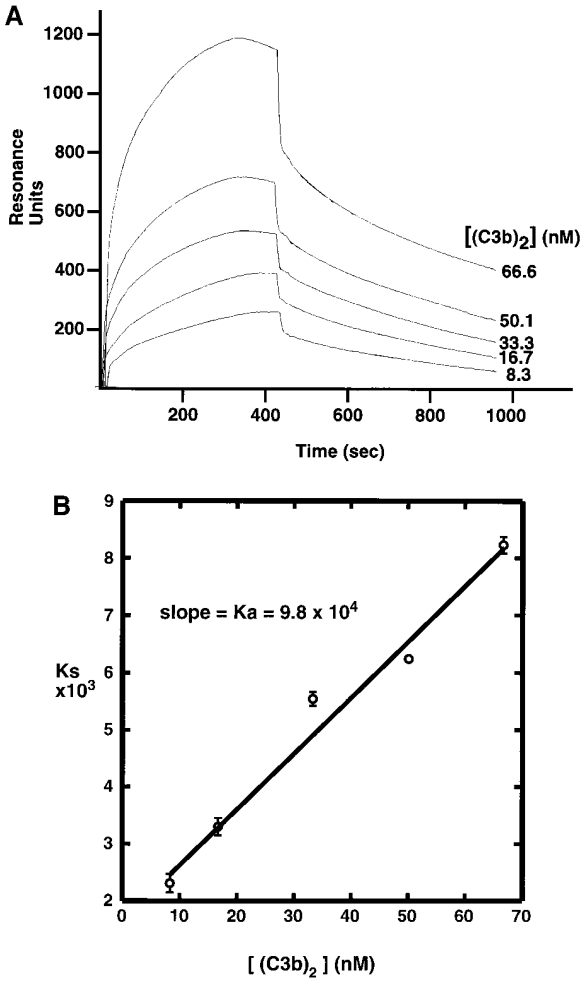

Figure 5. Kinetic Binding Data: C3b Dimers Bind to CR1, Documenting That Immobilized rsCR1 Is Functional

(A) Plot of resonance units (RU) versus time illustrating the observed association and dissociation of $(C3b)_2$. The association was monitored from 0 to 300 sec, and the dissociation occurred during the interval from 450 to 600 sec.

(B) Data from (A) were used to plot Ks versus $[(C3b)_2]$. The slope of the least squares regression line (r^2 = 0.977) corresponds to the apparent association rate constant.

the value of the reported equilibrium dissociation constant (K_{eq}) of monomeric C3b for CR1 (Arnaout et al., 1981). Further, the sequential binding of two identical binding sites would not significantly change the observed association kinetics (Gertler et al., 1996). At the time the dissociation phase begins, the $(C3b)_2$ may be bound monovalently or divalently; thus, a curve-fitting model assuming parallel dissociation of two distinct complexes was employed. The association and dissociation models closely fit the experimental data (not shown). Table 1 contains the observed rate constants, k_a and k_d, and the calculated equilibrium dissociation constant, K_{eq}, which corresponds to the ligand concentration at which half of the receptors are occupied. The K_{eq} for $(C3b)_2$ binding to CR1 was calculated for the five $(C3b)_2$ concentrations used and averaged 27.6 nM, with a range from 17.9 nM to 40.6 nM. An alternative analysis of the association kinetics, which does not assume a known k_d, yielded a k_a of 9.79×10^4 $mol^{-1} \times sec^{-1}$, an apparent k_d of 1.7×10^{-3} sec^{-1}, and a calculated K_{eq} of 17.4 nM (Figure 5B). Using the more reliable average k_{d2} determined from the dissociation kinetics, 2.078×10^{-3} sec^{-1} (Table 1), the calculated K_{eq} for $(C3b)_2$ binding to CR1 is 21.2 nM, in good agreement with the first method. These values are not significantly different from the previous determination of 9.5 nM for a similarly prepared $(C3b)_2$ (Arnaout et al., 1981), thus validating our methods.

Table 1. Kinetic Data for $(C3b)_2$ Binding to Immobilized CR1

$[C3b]_2$ nM	k_a (SE) $\times 10^{-4}$ $mol^{-1} \times sec^{-1}$	k_{d1} (SE) $\times 10^3$ sec^{-1}	k_{d2} (SE) $\times 10^3$ sec^{-1}	K_{eq1} (SE) μM	K_{eq2} (SE) nM
8.3	5.5 (1.8)	65.8 (15.8)	1.9 (0.9)	1.2 (0.49)	34 (19.8)
16.7	6.0 (0.9)	276 (11.1)	2.4 (0.04)	4.6 (0.71)	40 (6.0)
33.3	10.5 (0.3)	261 (14.2)	2.2 (0.05)	2.5 (0.15)	21 (0.77)
50.1	8.3 (0.2)	346 (46.6)	2.1 (0.05)	4.2 (0.57)	25 (0.85)
66.7	9.9 (0.2)	110 (17.7)	1.8 (0.4)	1.1 (0.18)	18 (4.0)
				$\bar{x}$ = 2.7 μM (1.06)	$\bar{x}$ = 27.6 nM (21.1)

In a similar analysis using five different concentrations of unlabeled C1q ranging from 6.5 to 52.2 nM, binding to CR1 was evident in normal ionic strength buffer (Figure 6A). No C1q binding was observed to a parallel channel that was not derivatized, or to a channel that was derivatized with C1q, data that demonstrated the specificity of C1q binding to rsCR1 in this assay. Using the same kinetic models as used for $(C3b)_2$ binding, a good fit to the experimental data was obtained. Analysis of the dissociation data for the more rapidly dissociating complex yielded an apparent k_d that ranged from 0.019 to 0.033 sec^{-1} among the five concentrations analyzed. The more slowly dissociating complex had an apparent k_d that ranged from 0.84 $\times$ 10^{-3} to 2.09 $\times$ 10^{-3} sec^{-1}.

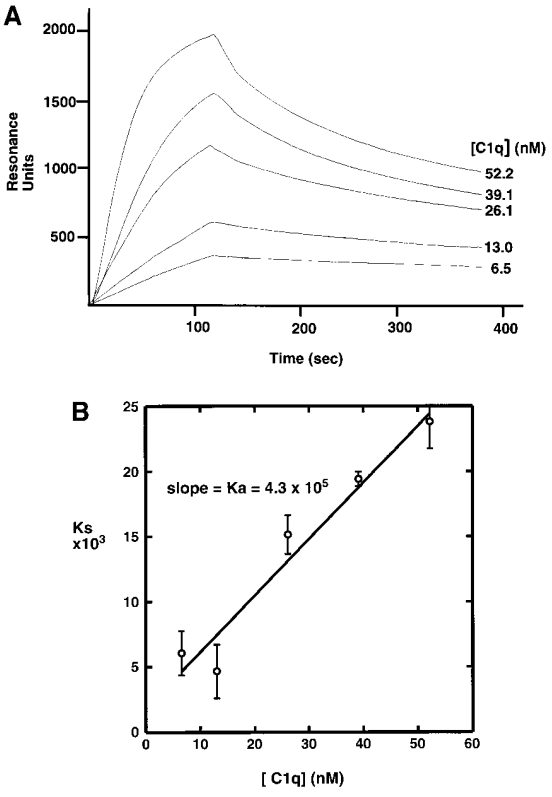

Figure 6. Kinetic Binding Data: C1q Binds to Immobilized rsCR1

(A) Plot of resonance units (RU) versus time illustrating the observed association and dissociation of C1q in normal saline. The association was monitored from 0 to 120 sec, and the dissociation occurred during the interval from 120 to 250 sec.

(B) Data from (A) were used to plot Ks versus [C1q]. The slope of the least squares regression line (r^2 = 0.947) corresponds to the apparent association constant, determined without assuming knowledge of the dissociation rate constant.

Using the latter dissociation constants in a simple association model, the apparent k_a ranged from 2.86 $\times$ 10^5 to 4.34 $\times$ 10^5 $mol^{-1} \times sec^{-1}$ (Table 2). The calculated K_{eq} for the slowly dissociating C1q–CR1 complex averaged 3.9 nM, with a range from 2.27 nM to 5.17 nM. An alternative analysis of the association kinetics, which does not assume a known k_d, yielded a k_a of 4.34 $\times$ 10^5 $mol^{-1} \times sec^{-1}$, an apparent k_d of 1.95 $\times$ 10^{-3} sec^{-1}, and a calculated K_{eq} of 4.49 nM (Figure 6B). Using the more reliable average k_d determined from the dissociation kinetics, 1.49 $\times$ 10^{-3} sec^{-1}, the calculated K_{eq} for C1q binding to CR1 was 3.43 nM, in good agreement with the first method. The most likely interpretation of these data is that the slowly dissociating complex interacted with CR1 via more of the six identical stems of C1q than did the rapidly dissociating complex. Importantly, C1q has a higher apparent affinity for CR1, under normal ionic conditions, than does $(C3b)_2$.

When C1q was directly immobilized to the chip by NHS/EDC chemistry, we were unable to detect rsCR1 binding in isotonic buffer (data not shown). In retrospect, this was probably because the C1q was denatured during the coupling reaction, which has been observed with other proteins (Gertler et al., 1996). However, when $C1q^{bio}$ was immobilized to a streptavidin chip, it was possible to detect CR1 binding, but only at relatively high concentrations of rsCR1 (435 nM to 3480 nM). The very rapid association and dissociation of rsCR1 to the immobilized $C1q^{bio}$ precluded an accurate determination of the rate constants by the BIAcore technique (BIA Evaluation and BIA Simulation, Pharmacia). The most likely explanation for why C1q bound at high apparent affinity to immobilized CR1, while soluble CR1 bound poorly to immobilized C1q, is that C1q has a higher valency for CR1 than CR1 has for C1q. For example, C1q with its six identical stems may have six binding sites for CR1, while CR1 apparently has fewer sites for binding to C1q.

Localization of a Binding Site in LHR-D for C1q

Using deletion mutations that previously had been employed to define the binding sites for C4b and C3b (Klickstein et al., 1988), we found that ^{125}I-C1q tails consistently bound to a fragment of rCR1 containing the nine SCR closest to the membrane. This region includes the fourth LHR, LHR-D (SCR 22–28) (Figure 7). Because all of the constructs studied contained all or part of LHR-D, we could not exclude additional binding sites in LHR-A, -B, or -C. Computer-aided sequence alignment (Wisconsin Package, Version 8, Sept. 1994, Genetics Computer Group, 575 Science Drive, Madison, WI 53711) revealed that the SCR in C1r and C1s are most

Table 2. Kinetic Data for C1q Binding to Immobilized CR1

[C1q] nM	k_a (SE) $\times 10^{-5}$ $mol^{-1} \times sec^{-1}$	k_{d1} (SE) $\times 10^3$ sec^{-1}	k_{d2} (SE) $\times 10^3$ sec^{-1}	K_{eq1} (SE) nM	K_{eq2} (SE) nM
6.5	3.7 (2.63)	23.6 (4.8)	0.84 (0.03)	64 (47.3)	2.3 (1.64)
13.0	2.7 (1.08)	22.3 (3.4)	1.24 (0.04)	83 (35.5)	4.3 (1.73)
26.0	4.3 (0.57)	23.7 (1.5)	1.54 (0.03)	55 (8.1)	3.6 (0.48)
39.0	4.3 (0.13)	19.1 (1.1)	1.74 (0.06)	44 (2.9)	4.0 (0.18)
52.0	4.0 (0.285)	32.7 (1.9)	2.09 (0.04)	82 (7.5)	5.2 (0.38)
				$\bar{X}$ = 59 nM (60.2)	$\bar{X}$ = 3.9 nM (2.47)

homologous to SCR of LHR-D, which is consistent with LHR-D containing a CR1 binding domain for C1q.

Discussion

CR1 (CD35) is a single-chain, integral membrane glycoprotein that has been recognized as the major cellular receptor for C4b and C3b (Fearon, 1980). The extracellular domain of the protein is comprised of SCR, which are characteristic of C3/C4 binding proteins (reviewed in Hourcade et al., 1989). The rsCR1 used in these studies is truncated after SCR 30. Groups of 7 SCRs in CR1 are further organized into 4 long homologous repeats (LHR-A, -B, -C, and -D) (Klickstein et al., 1987). LHR-A contains a C4b binding site, and LHR-B and LHR-C each contain a binding site for C3b (Klickstein et al., 1988; Krych et al., 1991), which also binds C4b, but at a lower affinity (Reilly et al., 1994). We have demonstrated C1q binding to CR1 by three different means: (1) equilibrium binding of labeled C1q to CR1-transfected K562 cells (Figures 2A and 2B); (2) equilibrium binding of labeled C1q (Figures 3 and 4) and labeled C1q tails (Figure 7) to plated rsCR1; and (3) real-time binding of unlabeled C1q to immobilized rsCR1 in normal saline (Figures 6A and 6B). Although there are likely other C1q binding sites on CR1, we consistently detected good binding of ^{125}I-collagen tails to a construct containing LHR-D (SCR-22–28) and the last two SCR of the extracellular domain, SCR 29 and 30. Interestingly, the region of CR1 most homologous to the SCR of C1r and C1s is LHR-D. Until now, there has been no binding function assigned to this region.

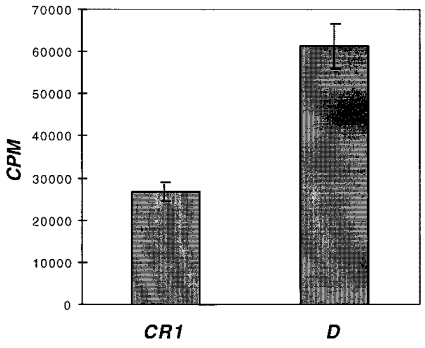

Figure 7. Binding of ^{125}I-C1q Collagen Tails to Immobilized Constructs of CR1

Microtiter wells were coated with anti-LHR-D (MAb 6B1, T Cell Sciences) at 3 μg/ml and blocked with milk. NP-40 lysates of equal numbers of control, and transfected CHO cells were added to allow the MAb to capture the constructs. Lysates of nontransfected CHO cells were added to some wells to allow determination of nonspecific binding. After washing wells, ^{125}I-C1q collagen tails (10 nM) were added for binding in 0.67 × PBS-Tween binding buffer (5.9 mSi). Results (mean ± SE, n = 4) are the specific binding, which has been normalized to equal molar amounts of constructs (see Experimental Procedures). This experiment was repeated using binding buffers of 3.75, 4.44, and 5.9 mSi with similar results.

The $(C3b)_2$ partially inhibited binding of C1q to immobilized CR1. One possible explanation for the partial inhibition is that the binding sites for the two ligands differ, but are close enough that $(C3b)_2$ sterically hinders C1q binding. Both $(C3b)_2$ and C1q are large molecules, ≈360,000 and 462,000 daltons, respectively. A second possibility is that there is more than one binding site for C1q that accounts for the partial inhibition of C1q binding by $(C3b)_2$. This would be consistent with our suspicion that there is an additional binding site for C1q outside of LHR-D (data not shown). Thirdly, the binding of $(C3b)_2$ may affect the tertiary structure of CR1 in a way that inhibits C1q binding. These three possibilities are not mutually exclusive.

Derivatization of ligands always risks introducing artifacts, and C1q is especially sensitive to manipulation. It was reported recently that $C1q^{bio}$, along with other biotinylated proteins, failed to show any specific binding to cells (Storm et al., 1996). We have found that in the case of C1q, its specific binding to PMN (Jack et al., 1994), CR1-transfected cells, and to plated CR1, and its residual hemolytic activity are adversely affected by high levels of biotinylation. However, with low levels of biotinylation, and also helped by the use of avidin/biotin blocking reagents (Vector Labs), $C1q^{bio}$ demonstrates specific binding to K-CR1 (Figure 2A). During the course of these experiments, we found that the hemolytic function of C1q was more sensitive to biotinylation than the binding site for CR1. Biotinylating with NHS-biotin at 0.6 μg/ml and 30 μg/ml yielded two preparations of $C1q^{bio}$ with near normal binding to CR1, but with 23% and 90% losses in hemolytic activity, respectively. Radiolabeling C1q has also been shown to increase the dissociation constant of C1r and C1s from C1q (Tseng et al., 1997). In our plate binding assay performed at 2/3 normal ionic strength (5.9 mSi), the 1/2 maximal binding of C1q (Figure 4A) and 1/2 maximal inhibition by unlabeled C1q (Figure 4B) were about 0.5 nM. Surface plasmon resonance analysis, on the other hand, allowed the direct measurement of binding by nonderivatized C1q in normal ionic strength buffer. The affinity values derived from the two methods are internally consistent, considering that C1q would be expected to have a lower binding affinity in the plasmon resonance analysis because of the adverse effect of a higher salt concentration on

binding to rsCR1 (Figure 3). It is interesting to note that both $C1r_2C1s_2$ binding and CR1 binding to C1q occur in the collagen domain (Figure 7) and both binding reactions are strongly inhibited by salt (Siegel and Schumaker, 1983; Tseng et al., 1997). CR1, C1r, and C1s all contain SCR units; therefore, we propose that there is a common binding site on the collagen stems of C1q for SCR-containing proteins. This hypothesis is supported by the observation that when $C1r_2C1s_2$ is bound to C1q, the C1q is unable to bind to C1qR-bearing cells (Tenner et al., 1980).

An intriguing question is why CR1 was not identified as a C1q receptor previously. While this may have to do with the low density of CR1 expression on resting cells (Arnaout et al., 1981; Fearon et al., 1983), the most likely explanation is that insolubilized C1q is a poor affinity ligand for CR1. If the K_{eq} for CR1 binding to immobilized C1q were in the range of 0.3–3 μM, where binding was observed to immobilized $C1q^{bio}$ in the BIAcore analysis (data not shown), then the CR1 concentration in cell lysates would be far too low to detect specific binding. The presence of detergent used to solubilize cell membranes might further impair binding. The probable explanation for why CR1 binds poorly to immobilized C1q, while C1q binds at high apparent affinity to immobilized CR1, is that C1q has a higher valency for CR1 than CR1 has for C1q. For example, C1q with its six identical stems may have six binding sites for CR1, while CR1 has fewer sites for binding to C1q. Consistent with this hypothesis, CR1 on the surface of cells is highly clustered (Petty et al., 1980; Edberg et al., 1987; Paccaud et al., 1988, 1990; Chevalier et al., 1989), which would allow one molecule of C1q to interact with multiple CR1 molecules.

The role of CR1 with respect to other molecules proposed as C1q receptor will have to be defined. Calreticulin has been proposed as a C1q receptor, but it is primarily found in the ER, where it acts as a Ca^{++}-binding protein and as a chaperonin for nascent proteins. Perhaps as a consequence of this chaperone function, calreticulin is also found in the granule fraction of some cells (reviewed in Bleackley et al., 1995) and might be expressed on cells as a consequence of degranulation and the adsorption to plasma membrane (Eggleton et al., 1994). It is unclear how such adsorbed calreticulin could act as a receptor. Recent work indicates that calreticulin preferentially binds to the globular domain, as opposed to the collagen domain of C1q (Kishore et al., 1997). These data have not been reconciled with the putative role of calreticulin as the C1q receptor for the collagen domain. Antibodies to a recently cloned 126k M_r transmembrane protein block the C1q-stimulated phagocytosis by monocytes (Guan et al., 1991; Nepomuceno et al., 1997). No binding of C1q has been demonstrated to this novel 126k M_r protein; thus, it may participate as an element of a larger C1q receptor complex. If the amino-terminal C-type lectin domain of this transmembrane protein were responsible for binding C1q, one would expect that C1q binding to cells would be Ca^{++}-dependent, which was not observed (Jack et al., 1994).

Although C1q has many biological effects presumably mediated through cell surface receptors, (reviewed in Ghebrehiwet et al., 1993; Tenner, 1993), there is little known about how CR1 might mediate transmembrane signaling. CR1 participation in the cellular responses to C1q might require that other molecules be recruited. There is precedence for CR1 associating with other molecules: CR1 is known to associate with CD21 in the membrane of B cells (Tuveson et al., 1991; Matsumoto et al., 1993), and when CR1 is cross-linked, it forms attachments to the cytoskeleton (Jack et al., 1986; Brown, 1989). Finally, CR1 may not be the only C1q receptor, since C1q reportedly does bind and activate some cells, e.g., endothelial cells (Zhang et al., 1986; Lozada et al., 1995), which do not express detectable CR1 (Shaw et al., 1995).

There are important biological implications for C1q/CR1 binding. CR1 on erythrocytes is critical for the transport of immune complexes and their subsequent clearance. Thus, CR1 in humans may be solely responsible for clearing immune complexes opsonized by complement, whether C1q and/or C3b/C4b. That C1q might participate in the clearance of immune complexes may explain several clinical observations. It is well recognized that deficiency of an early component of the classical pathway predisposes to autoimmune diseases, typically SLE (Lachmann, 1984; Atkinson, 1986). Only about half of the patients with homozygous C2 deficiency have lupus-like disease characterized primarily by cutaneous manifestations, and vital organ involvement is uncommon (Colten et al., 1992). In contrast, C1q deficiency is almost invariably associated with SLE (31/33 patients) (Petry et al., 1997), and the disease is very severe, often including central nervous system pathology, glomerulonephritis, and the presence of autoantibodies (Bowness et al., 1994). The clinical manifestations of homozygous C4 deficiency are intermediate in severity between those associated with C2 and C1q deficiency (Colten and Rosen, 1992). Deficiency of C2 would result in defective opsonization by C3 only, while deficiency of C4 would result in defective opsonization by both C4 and C3. However, immune complexes might still be effectively opsonized by C1q. In C1q deficiency, however, neither C1q, C3b, nor C4b would effectively bind to immune complexes, and the complexes might then be deposited in vital organs such as the kidney rather than the normal targeting to liver and spleen for clearance (reviewed in Hebert, 1991). An alternate hypothesis places the critical role of C1q in preventing autoimmune disease before the formation of immune complexes. In a recently published study, C1q has been shown to directly bind to the blebs of apoptotic keratinocytes (Korb et al., 1997). Because these blebs are enriched in nuclear antigens recognized by autoantibodies in SLE, it is hypothesized that C1q is normally involved in the clearance/tolerance of these antigens.

The mechanism underlying the severe autoimmune disease associated with C1q deficiency may be due to the inability to activate the classical pathway or the inability to recruit C1q receptor–bearing cells. Now that CR1 has been identified as a C1q receptor, it will be possible to address these alternatives.

Experimental Procedures

Buffers/Reagents

PBS used in microtiter well assays: 0.15 M NaCl, 0.05 M sodium/potassium phosphate (pH 7.4). PBS used in plasmon resonance

assays: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 (pH 7.3). The C1q binding buffer used with transfected cells was "HBSS/2" = 1 vol HBSS without Ca^{++}/Mg^{++}:1 vol 5% glucose-0.2% gelatin solution). The C1q binding buffer used in the microtiter plate assay was 0.67 × PBS-Tween = 67% PBS, 33% dHOH, 0.05% Tween-20, 5.9 mSi at 0°C). The following reagents were purchased as noted: 1,3 diaminopropane (Sigma Chemical, St. Louis, MO), dinonyl phthalate (Arcos, Geel, Belgium), dibutyl phthalate (Sigma). Recombinant soluble human CR1 (rsCR1) was kindly provided by Drs. Una Ryan and Henry Marsh of T-Cell Sciences, Needham, MA.

Complement C1q and Collagen Tails of C1q

C1q for biotinylation (as used in Figure 2A) was isolated from human serum by a procedure using BioRex 70, as originally described (Tenner et al., 1981b) and modified (Jack et al., 1994). Each batch of $C1q^{bio}$ was tested for functional activity (Tenner et al., 1981b). Because of the propensity of the native C1q made by the BioRex method to aggregate, an alternative isolation method for C1q, utilizing fractional euglobulin precipitation and gel permeation chromatography, was devised to prepare C1q for radiolabeling. In brief, 30 ml of fresh serum, 5 mM EDTA was dialyzed against a euglobulin precipitation buffer (20 mM morpholine ethane sulfonic acid, 5 mM benzamidine, 0.5 mM EDTA [pH 6.5]) in tubing for 16 hours at 4°C. The precipitate was collected by centrifugation (8000 × g, 10 min), washed twice in 5 mM propanediamine, 0.05 mM EDTA (pH 8.8) (PDE buffer) (Liberti et al., 1981), then dissolved in 1.4 ml of PDE buffer supplemented with 300 mM NaCl (PDE/NaCl), 0.5 mM PMSF. The mixture was centrifuged at 10,000 × g for 5 min to remove undissolved precipitate. The supernatant containing C1q was aliquoted (0.45 ml) to each of three microfuge tubes. Cold water (1 ml) was added to each tube, and after 10 min at 4°C, the resulting precipitates were collected by centrifugation (10,000 × g, 5 min). The precipitates in each tube were dissolved in 75 μl of PDE/NaCl buffer, and then 85 μl of 2 × PBS was added. This material was recentrifuged (15,000 × g, 5 min), and 150 μl was applied to a TSK $G4000SW_{XL}$ column (Supelco, Bellefonte, PA) equilibrated in 2 × PBS, 0.5 mM EDTA at a flow rate of 0.5 ml per min. C1q eluted as a peak with a retention time of 18.6 min consistent with a MW of 462 kDa), and the peak was collected by hand. Analysis of the C1q revealed the distinct a, b, and c chains by SDS–PAGE with silver staining. The specific activity of the C1q was 400 hemolytic units (Z) per μg of protein using the assay based on the BioRex drop through fraction of human serum (Tenner et al., 1981b). Protein was assayed by the micro BCA (bicinchoninic acid method; Pierce Chemicals, Rockville, IL), and C1q concentrations were calculated from a BSA standard curve.

The collagen domain of C1q was prepared from a pepsin digest of C1q (Siegel and Schumaker, 1983) and purified by HPLC gel permeation chromatography using the same TSK $G4000SW_{XL}$, vide supra. Protein was quantified with the BCA assay, using BSA as a standard.

Biotinylation of C1q

In a typical biotinylation reaction, 840 μg of C1q was reacted with 4.3 μg of NHS-biotin (Pierce Chemicals, Rockville, IL) in 1 ml of PBS for 30 min at room temperature with intermittent agitation. The reaction was stopped by the addition of concentrated ethanolamine to give a final concentration 0.1 M. The reaction mixture containing $C1q^{bio}$ was subsequently dialyzed against 120 mM KCL, 10 mM Tris/HCl (pH 7.4) and the protein assayed. We were not able to determine the final biotin:C1q ratio using the reagents provided with the biotinylation kit, but it was necessary to biotinylate "lightly" to avoid aggregation. Each batch of $C1q^{bio}$ was tested for functional activity (Tenner et al., 1981b).

Radioiodinated C1q and C1q Collagen Tails

Glucose oxidase (Sigma) and lactoperoxidase (Sigma) were separately coupled to beads of cross-linked bis-acrylamide/azlactone copolymer beads (3M Emphaze; Pierce Chemicals, Rockville, IL) according to the manufacturer's instructions. For both enzymes, the coupling ratio was 1 mg of protein per hydrated equivalent of 24 mg of dried beads. The optimal ratio (1/4) of coupled glucose oxidase to coupled lactoperoxidase was determined in a preliminary experiment by combining the beads in different ratios and measuring the resultant enzymatic activity using 0.1% D-glucose in tetramethylbenzidine solution (Kirkegaard and Perry, Gaithersburg, MD) as the substrates. The development of blue color was followed spectrophotometrically.

At the time of radiolabeling, glucose oxidase beads (3 μl of a slurry) and lactoperoxidase beads (12 μl of a slurry) were washed into PBS. Sodium-^{125}Iodine (3 μl, carrier free, 100 mCi/ml, New England Nuclear, Boston, MA) was added to the bead pellet, followed by 70 μl of C1q (400 μg/ml in 2 × PBS), 70 μl of dHOH, and 10 μl of glucose solution (100 μg/ml PBS). The reaction proceeded for 20 min at room temperature with intermittent shaking. The reaction supernatant was applied to a PD-10 gel filtration column (Pharmacia), which had been equilibrated in PBS, 0.1% gelatin. The radiolabeled C1q was pooled and characterized. 98% of CPM of the ^{125}I-C1q were precipitable with 10% TCA. ^{125}I-C1q was quantified by a sandwich ELISA using anti-C1q MAb (Quidel, San Diego, CA) as the capture antibody and goat anti-human C1q (IncStar, Stillwater, MD) as the indicator antibody. The reaction was developed with horseradish peroxidase–conjugated rabbit anti-goat IgG (IncStar) and tetramethylbenzidine substrate. The color reaction was stopped by the addition of H_3PO_4, and the OD_{450} was quantified using an ELISA plate reader (Molecular Devices, Menlo Park, CA). C1q of a known protein concentration was used as a standard. Multiple lots of C1q were iodinated with specific activity ranging from 5×10^5–1×10^6 CPM per μg of C1q as quantified by ELISA, and the functional activity was 400 hemolytic units (Z) per μg.

Collagen tails of C1q were radioiodinated using IodoGen (Pierce Chemicals, Rockville, IL) to a specific activity of 2.4×10^5 cpm/μg of protein.

Preparation of $(C3b)_2$

C3 purified from fresh human plasma by standard methods (Hammer, 1981, #248) was treated with trypsin to produce C3b (Fearon, 1983, #638). The trypsin was inactivated by addition of diisopropylfluorophosphate and the C3b purified by chromatography on Sephacryl S300 in PBS. The fractions containing C3b were pooled, concentrated to 1.4 mg/ml (Centriprep, Amicon, Beverly, MA), and stored at 4°C for 3 weeks to allow formation of dimers via oxidation of the free sulfhydryl group, as occurs in stored C3 (Arnaout et al., 1981). $(C3b)_2$ was separated from monomeric C3b by gel filtration on Sepharose CL-2B in PBS (Pharmacia LKB Biotechnology, Piscataway, NJ), and the expected M_r was confirmed by SDS–PAGE. Peak fractions were pooled, aliquoted, and stored at −80°C.

Preparation of K562 Cells with Cell Surface CR1

The plasmid pBSHyg, which directs the expression of hygromycin resistance, was prepared by ligation of the 2.0 kb HindIII–NruI fragment from REP3 (Groger et al., 1989) into the HindIII–HincII sites of Bluescript KS(−) (Stratagene, La Jolla, CA). The plasmid paABCD directs the expression of the F allotype of human CR1 (Klickstein et al., 1988). K562 cells were electroporated (250 V, 960 μF) with 200 ng of pBSHyg linearized with XmnI with or without 20 μg of paABCD linearized with SfiI. Transfectants were selected by culture in RPMI with 20% FCS supplemented with hygromycin at 200 μg/ml for 2 weeks, then transferred to RPMI with 10% FCS. K562 cells transfected with pBSHyg alone were termed K-Hyg, and those transfected with paABCD were termed K-CR1. The K-CR1 cells were immunopanned (Wysocki et al., 1978) on immobilized anti-CR1 MAb (YZ-1) (Changelian et al., 1985) to select a uniformly positive population of cells. The CR1 expression on the control K-Hyg and K-CR1 transfectants was assessed by YZ-1 and FACs analysis, vide infra. Once during the period of experimentation, the K-CR1 transfectants were reselected by panning using YZ-1 to enhance CR1 expression. Western blotting of transfected cells to detect CR1 was performed as described (Yoon and Fearon, 1985). For the adhesion assay, $(C3b)_2$ was adsorbed to plastic wells in 50 mM Tris buffer (pH 9), and the plate was blocked with 1% BSA. K562 transfectants were added and allowed to bind for 30 min at room temperature. After gently washing the plates, the adherent cells were enumerated using light microscopy.

FACS Analysis

A FACStar (Becton Dickinson, San Jose, CA) was used, and 10^4 cells were analyzed for each variable. Transfected cells were assessed for

CR1 expression using the murine MAb YZ-I and FITC-goat anti-mouse IgG (Tago-Biosource, Camarillo, CA).

^{125}I-C1q Binding to Transfected Cells

Binding was performed in a polypropylene microfuge tube using "HBSS/2" (HBSS without Ca^{++} and Mg^{++}), which was diluted with an equal volume of 5% glucose-0.2% gelatin solution. The total reaction volume of 0.33 or 0.44 ml contained 5×10^6 cells/ml and increasing amounts of ^{125}I-C1q (specific activity: 1.1×10^6 cpm/ μg). The binding reaction proceeded at room temperature for 45 min with regular agitation. Aliquots (0.1 ml) of the reaction mixture were removed and layered onto 300 μl of an oil mixture (85% dibutyl phthalate, 15% dinonyl phthalate) in microfuge tubes (0.4 ml of polyethylene, #1404–1000, USA/Scientific Plastics, Ocala, FL). The tubes were spun for 2 min at 9000 $\times$ g (Microfuge B, Beckman Instruments, Fullerton, CA), and the tips containing the cell pellets were cut off and counted in a gamma counter.

C1q^{bio} Binding to Transfected Cells

Cells were first reacted with avidin/biotin blocking reagents following the manufacturer's instructions (Vector Laboratories, Burlingame, CA) for 10 min at room temperature. Subsequently, 5×10^6 cells were aliquoted to tubes and HBSS/2 buffer $\pm$ dilutions of C1q^{bio} were added (final volume, 135 μl) and incubated for 30 min at 37°C. After appropriate washes, FITC-avidin (Vector)–diluted 1/250 was added for 25 min at room temperature. All later wash and incubation steps were performed in the same HBSS/2 buffer. After a wash, the cells were fixed in 1% paraformaldehyde/PBS and analyzed by FACS.

C1q Binding to Insolubilized rsCR1

Microtiter wells (Immulon 1 Removawell strips, Dynatech Labs, Alexandria, VA) were treated with 0.1 ml of rsCR1 (5 or 8 μg/ml, both saturating concentrations) diluted in coating buffer (0.01 M Na_2CO_3, 0.04 M $NaHCO_3$ (pH 9.6) for 2 hours at 37°C or overnight at 4°C. For the C1q^{bio} binding studies, wells were blocked with SuperBlock (Pierce Chemicals, Rockville, IL), per the manufacturer's instructions. For binding studies using ^{125}I-C1q or ^{125}I-collagen tails, blocking was done for 2 hr at 37°C with 3% nonfat dried milk (BioRad, Hercules, CA), 0.5% Tween-20 (Baker Chemical, Phillipsburg, NJ) in PBS.

Varying amounts of ^{125}I-C1q in 0.67 $\times$ PBS-Tween binding buffer were incubated in the rsCR1-coated wells for 45 min at room temperature in the presence or absence of a competitive ligand. Total reaction volume was always 100 μl. After the binding incubation, the wells were emptied by aspiration and then the plate was turned upside down and vigorously slapped five times against layers of filter paper. Subsequently, the wells were separated and individually counted in a gamma counter.

C1q Binding to Insolubilized Deletional Mutants of CR1

Microtiter wells were coated with MAb anti-LHR-D (3 μg/ml) (clone 6B1, gift of Dr. Henry Marsh, T-Cell Sciences, Needham, MA) as described for CR1 above. Clone 6B1 binds an epitope within the region of SCR 26–30 (data not shown). Wells were blocked with dried milk as described above. NP-40 lysates of control CHO cells, or CHO cells that were transfected with either the full-length CR1 (piABCD) or LHR-D (piD) (Klickstein et al., 1988) were added to the wells for 2 hr at room temperature to allow the immobilized anti-LHR-D to capture the recombinant CR1 antigen. After washing the wells three times with binding buffer (vide supra), ^{125}I-collagen tails (10 nM) in binding buffer were added for 45 min at room temperature. After two washes in binding buffer, the wells were separated and individually counted in a gamma counter. To normalize the binding data, compensation was made for the molar amount of LHR-D antigen in each lysate. A two-site capture radioimmunoassay was performed that used a polyclonal antibody to capture the antigen and MAb ^{125}I-6B1 for detection. 6B1 has a single epitope in CR1 (LHR-D), unlike most other anti-CR1 MAbs.

Biospecific Interaction Analysis

Binding of native C1q to immobilized sCR1 was analyzed using a BIAcore instrument (Pharmacia). sCR1 (800 μg/ml in 10 mM citrate [pH 4.8] buffer $\times$ 50 μl at a flow of 5 μl/ min) was coupled to a CM5 sensor chip (Pharmacia) using EDC and NHS according to the manufacturer's instructions (Johnsson et al., 1991). These conditions resulted in 8852–9150 RU stably coupled in five separate immobilizations. Binding studies were performed in PBS at 25°C using a flow rate of 5 μl/min. For analysis of $(C3b)_2$ binding, the immobilized rsCR1 was regenerated for subsequent analysis by washing with 0.1 M sodium citrate (pH 5.0). In C1q binding studies, the CR1 was regenerated by washing with 0.5 M NaCl. Data was analyzed using BIAcore Incorporated software (BIA Evaluation and BIA Simulation, Pharmacia).

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