GENERATION OF THREE DIFFERENT FRAGMENTS OF BOUND C3 WITH PURIFIED FACTOR I OR SERUM

II. Location of Binding Sites in the C3 Fragments for Factors B and H, Complement Receptors, and Bovine Conglutinin*

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The third component of complement (C)[1] has a central role in the activation of both the classical and alternative pathways. C3 also has a variety of different functions that are expressed either after C3 activation or during the normal breakdown of the molecule. These functions of C3 are dependent upon the exposure within C3 of binding sites for other serum proteins or for membrane C3 receptors. None of these binding sites is available in native C3 (1, 2), and each type of binding site is exposed only during C3 activation and subsequent fragmentation. The structure and function of the different C3 fragments and the mechanism of their generation has been a subject of intense research after initial reports that inactivated C3b (iC3b) expressed functions that were distinct from those of C3b (3, 4). Recent studies have now demonstrated that factor I is the primary enzyme responsible for the normal physiologic breakdown of C3b (5–7). The key to defining the pathway of I cleavage of C3b was the demonstration that two different cofactors are required for I cleavage of C3b and iC3b (6,

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[1] *Abbreviations used in this paper:* ATS, activated thiol Sepharose; B, factor B; BDVA, 3.5 mM veronal buffer containing 10 mM sodium chloride, 1% BSA, 3.5% dextrose, 0.2% sodium azide, and 2 mM each of calcium chloride and magnesium chloride; BDVEA, calcium-magnesium-free BDVA with 20 mM EDTA; BSA, bovine serum albumin; C, complement; C3-ms, C3 fragment-coated fluorescent microspheres, C3d-OR, soluble C3d-sheep erythrocyte membrane complexes; CR_1, C receptor type 1, the C3b receptor; CR_2, C receptor type 2, the C3d receptor; CR_3, C receptor type 3, iC3b receptor; D, factor D; DGVB, dextrose gelatin veronal buffer; EC3, erythrocyte-C3 complexes; EDTA, ethylene diamine tetraacetate; EGTA, ethyleneglycol-bis-(beta-amino-ethyl ether) N,N'-tetra acetic acid; GVB, gelatin veronal buffer; H, factor H (formerly β1H globulin); HBSS, Hanks balanced salt solution; I, factor I (formerly C3b-inactivator); K, bovine serum conglutinin; NADG, *N*-acetyl-D-glucosamine; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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7). H is the cofactor for I cleavage of fluid-phase C3b (8, 9), whereas erythrocyte (E) membrane CR_1 is the cofactor for I cleavage of fixed iC3b into fixed C3d,g and fluid-phase C3c (6, 7). Even though CR_1 may also serve as cofactor for I cleavage of fluid C3b (10), CR_1 probably can not replace H as the control protein of the alternative pathway, as patients with inherited H deficiency (11) have unregulated C3 activation resembling patients with I deficiency (12).

In the present investigation, the C3 fragments resulting from activation and breakdown by I and trypsin were examined for binding sites for six different C3-ligands: factors B and H, CR_1, CR_2, CR_3, and conglutinin (K). Evidence was obtained for at least four distinct binding sites in the C3 fragments for these six C3 ligands.

Materials and Methods

Buffers. Assays were performed in either isotonic or low ionic strength buffers containing either divalent cations, EDTA, or EGTA. Isotonic buffers included: Hanks balanced salt solution (HBSS) containing 1% BSA and either 0.6 mM calcium and 0.9 mM magnesium or 20 mM EDTA; and gelatin veronal buffer (GVB) containing either 0.15 mM calcium and 0.5 mM magnesium or 0.15 mM nickel. The low ionic strength buffers used were: dextrose gelatin veronal buffer (DGVB, 4 mS at 22°C) containing either 0.15 mM calcium and 0.5 mM magnesium or 0.15 mM nickel; 3.5 mM veronal buffer containing 10 mM sodium chloride, 1% BSA, 3.5% dextrose, 0.2% sodium azide, and 2 mM each of calcium chloride and magnesium chloride (BDVA, pH 7.2 and 4 mS at 22°C); and calcium-magnesium-free BDVA with 20 mM EDTA (BDVEA, pH 7.2 and 6 mS at 22°C).

Leukocytes and Erythrocytes (E). Blood from normal volunteers was drawn into acid citrate dextrose anticoagulant. After sedimentation of erythrocytes with dextran, mononuclear cells and neutrophils were separated by centrifugation on a two-step (d = 1.08 and 1.105) density gradient of Ficoll-Hypaque as previously described (13). Erythrocytes, taken from the dextran-sedimented fraction, and Raji lymphoblasts, cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum, were each washed three times in phosphate-buffered saline (PBS) before assay of C receptors. Each cell type was suspended at 4×10^6/ml in EDTA/HBSS/BSA, and maintained at room temperature until examined for C receptors. Soybean trypsin inhibitor (Sigma Chemical Co, St Louis, MO) at 1 mg/ml was added to all neutrophil preparations to inhibit elastase activity (13). Where indicated, portions of each cell type were resuspended before C receptor assay at 4×10^6/ml in either HBSS/BSA, BDVA, or BDVEA.

C Components, Trypsin, and Serum Reagents. C3, B, D, H, and I were isolated as previously described (6, 14). Trypsin was obtained from Sigma Chemical Co. Isotonic and low ionic strength (4 mS at 22°C) heat-inactivated serum reagents were prepared as before (6).

Purification of C3 Fragments. C3b, iC3b, C3c, C3d,g, and C3d were prepared on Activated Thiol Sepharose (ATS, Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with 10 mM cysteine, pH 7.0 as previously described (6). ATS-iC3b was prepared by H and I treatment of ATS-C3b in isotonic salt buffer, and contained no trace of C3b or C3d,g on SDS-PAGE. ATS-C3d,g, prepared by H and I treatment of ATS-C3b at low ionic strength (1.4 mS), contained ~10% iC3b (6). To remove iC3b from one C3d,g preparation, the C3d,g was absorbed with agarose coupled to monoclonal anti-C3c (clone 4) (15). Two C3c preparations were used, one generated by elastase treatment of ATS-C3b, and the other by H and I treatment of ATS-C3b in 1.4 mS buffer (6).

Preparation of C3-Coated Fluorescent Microspheres. Fluorescent microspheres (Covaspheres, Covalent Technology Corp, Ann Arbor, MI) were coated with iC3b, C3d,g, and C3c as previously described (13). Control BSA-coated microspheres were always prepared in parallel.

Formation of Sheep Erythrocyte-C3 Complexes (EC3) and Quantitation of The Different Fixed

C3 Fragments With ^{125}I-Labeled Monoclonal Antibodies Specific For C3c, C3g, and C3d. EC3b was prepared by two cycles of C3 addition, first with trypsin, and then with a bound nickel-stabilized C3-convertase formed with B and D (6). EC3bi was prepared by treatment of EC3b for 15 min at 37°C with isotonic heat-inactivated serum, 1.0 ml per 1×10^9 EC3b (6). EC3d,g was prepared by treatment of EC3b for 60 min at 37°C with 4 mS heat-inactivated serum, 1.0 ml per 1×10^9 EC3b (6). EC3d was prepared from EC3bi or EC3d,g by incubation for 30 min at 37°C at 1×10^9/ml in PBS containing 10 μg/ml of trypsin (6). EC3 were prepared with different amounts of C3 and the amount and type of bound C3 fragments were quantitated by assay for uptake of ^{125}I-labeled monoclonal antibodies specific for C3c, C3g, and C3d (6). One anti-C3c IgG (kindly donated by Dr. Charles Parker, Duke University, Durham, NC) was purified from ascites fluid obtained from Bethesda Research Laboratories (Gaithersburg, MD) by column chromatography on DEAE cellulose. A second anti-C3c IgG, clone 4, (15) was isolated from ascites fluid (made pH 5 with acetic acid) by precipitation with 5% caprylic acid (Sigma Chemical Co.) followed by precipitation of the caprylic acid supernatant with 50% saturated ammonium sulphate. Clone 9 anti-C3g (15) and clone 3 anti-C3d (15) were isolated in a similar way. Each anti-C3 was iodinated to a specific activity of 0.7–2.5 μCi/μg IgG using Iodogen (Pierce Chemical Co., Rockford, IL) (16).

In the first step of C3 addition with trypsin forming $EC3b_T$, the amount of C3 and trypsin (6) were adjusted for maximum C3b fixation without further trypsin cleavage of the bound C3b. Any cleavage of fixed C3b on the $EC3b_T$ was detected with ^{125}I-anti-C3g, that bound only to fixed iC3b and to fixed C3d,g and not to fixed C3b (6, 17). The analysis of a typical preparation of EC3 is given in Table I. Conversion of EC3b to EC3bi required 5 min at 37°C, and was determined to be complete when the uptake of ^{125}I-anti-C3g was equivalent to the uptake of ^{125}I-anti-C3c and ^{125}I-anti-C3d. Incomplete conversion to iC3b was indicated if the uptake of ^{125}I-anti-C3g was less than the uptake of ^{125}I-anti-C3c and ^{125}I-anti-C3d, whereas over-cleavage of iC3b to C3d,g was indicated when the uptake of ^{125}I-anti-C3c was less than the uptake of ^{125}I-anti-C3g and ^{125}I-anti-C3d. These conclusions derived with monoclonal antibodies were initially confirmed by SDS-PAGE and autoradiography of EC3 prepared with ^{125}I-labeled C3 (6). EC3d,g always contained 5% factor I-resistant C3b and iC3b, as indicated both by uptake of ^{125}I-anti-C3c (Table I) and by SDS-PAGE autoradiography (6). EC3d contained ~3% contaminating C3b, but no detectable iC3b. This was indicated both by the uptake of ^{125}I-anti-C3c in the absence of significant uptake of ^{125}I-anti-C3g (Table I) and by SDS-PAGE autoradiography of EC3d prepared with ^{125}I-labeled C3. (6).

Anti-CR_1, Anti-H, and Anti-Mac-1. Rabbits were immunized with purified CR_1 (10, 18) or H (14), and the $F(ab')_2$ fragments of the IgG antibodies were isolated (18). Analysis of

TABLE I

Detection And Quantitation Of Different C3 Fragments on EC3 With ^{125}I-Labeled Monoclonal Antibodies Specific for C3c, C3g, and C3d

EC3 type	Molecules ^{125}I-anti-C3 bound per EC3		
	Anti-C3c	Anti-C3g	Anti-C3d
$EC3b_T$*	5.7×10^3	1.3×10^2	5.4×10^3
EC3b‡	3.3×10^4	2.1×10^2	3.5×10^4
EC3bi§	2.8×10^4	2.7×10^4	2.8×10^4
EC3d,g§	8.1×10^2	2.5×10^4	2.6×10^4
EC3d§	7.1×10^2	2.4×10^1	2.3×10^4

* $EC3b_T$: prepared by trypsin activation of 2 mg of C3 in the presence of 1×10^{10} sheep E.

‡ EC3b: prepared by addition of 100 μg C3 per 1×10^9 $EC3b_T$,Bb,Ni^{++} (see Materials and Methods).

§ EC3bi, EC3d,g and EC3d were prepared from EC3b (see Materials and Methods).

the anti-H by immunodiffusion and immunoelectrophoresis demonstrated a single precipitin line of identity between purified H and human serum. In addition, the anti-H did not contain anti-C3 detectable by assay for agglutination of EC3b nor anti-CR_1 detectable by assay for inhibition of human E rosette formation with EC3b. Tissue culture supernatant containing 100 μg/ml of anti-Mac-1 antibody (19) was kindly provided by Dr. T. Springer (Sidney Farber Cancer Institute, Boston, MA).

Soluble C3d-Sheep E Membrane Complexes (C3d-OR). The membranes of 6×10^9 EC3d bearing 7.1×10^4 C3d per E, and only 3.6×10^3 C3b and 7.1×10^1 iC3b per E, were produced by lysis of the EC3d in 5 mM phosphate buffer, and then were solubilized with 0.25% Nonidet P40 as described by Fearon et al. (20) for C3b-OR. After removal of detergent with Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) the C3d-OR were concentrated to 1.2 ml, dialyzed against BDVA, and stored at 4°C.

Assays For Factor B Binding To C3. Purified B was examined for binding to C3b and iC3b that were covalently attached to ATS or sheep E. 1-cm × 15-cm columns containing either ATS-C3b or ATS-iC3b (3.4 mg C3b or iC3b/ml ATS) (6) were equilibrated with 3.5 mM veronal buffer, pH 7.2 containing either 0.5 mM magnesium chloride or 0.15 mM nickelous chloride (~1 mS at 22°C). Binding of 5.0 mg of B passed through these two columns was assessed by measuring B hemolytic activity in 1-ml fractions eluted at 20 ml/h at 4°C. The B contained 10% contamination with hemopexin that was monitored in column fractions by Ouchterlony assay. B titrations were performed with EC3b (1×10^8/ml) in DGVB containing 0.15 mM nickelous chloride, (DGVB-Ni). 25 μl dilutions of B in DGVB-Ni were mixed with 25 μl of D (200 ng/ml) and 25 μl of EC3b in V-bottom microtiter plates and incubated with agitation for 3 min at 37°C. Next, 25 μl of 80 mM EDTA, pH 7.2 and 25 μl of guinea pig C diluted 1:15 in 40 mM EDTA-GVB were added in succession, and incubation continued with agitation for 45 min at 37°C. To assess B binding to EC3bi, this assay was carried out with EC3bi instead of with EC3b.

Assay for H Binding to EC3b and EC3bi. H (2 mg) was labeled with 0.5 mCi ^{125}I using Iodogen (16), and had a specific activity of 0.05 μCi/μg. Increasing volumes of the [^{125}I]-H (750 μg/ml in PBS) were added to duplicate 10-mm × 75-mm plastic tubes containing 1×10^8 sheep E, EC3b, or EC3bi in either GVB or DGVB to give a total volume of 200 μl. After incubation for 30 min at 30°C, 100 μl (5×10^7 cells) was layered onto 1.0 ml of a 7:3 mixture of n-butyl phthalate and dinonyl phthalate (Sigma Chemical Co.) in a 1.5-ml tube and centrifuged for 45 s at 8,000 *g*. The supernatant containing the free [^{125}I]-H was then aspirated and the amount of EC3-bound [^{125}I]-H was determined by cutting off the bottom of the tube just above the pellet and counting it in a gamma counter (23). The nonspecific uptake of [^{125}I]-H by E was subtracted from the total [^{125}I]-H uptake observed with EC3 to determine the specific uptake of [^{125}I]-H. The concentration of free unbound [^{125}I]-H was calculated by subtraction of the specifically bound [^{125}I]-H concentration from the total [^{125}I]-H concentration in the assay mixture. The H-binding affinity was determined from the slopes of Scatchard plots derived by plotting the ratio of bound/free H concentrations versus the concentration of bound H (24).

Assay of Anti-H and anti-CR_1 for Inhibition of 4 mS Serum-mediated C3c Release from EC3bi. 1.0-ml volumes of low ionic strength heat-inactivated serum (6) were mixed with 250 μl of either PBS, $F(ab')_2$ anti-H (18.1 mg/ml), $F(ab')_2$ anti-CR_1 (1.65 mg/ml), or a mixture containing $F(ab')_2$ anti-H and $F(ab')_2$ anti-CR_1 (18.1 mg/ml and 1.65 mg/ml respectively). The amount of anti-CR_1 used was shown to be sufficient to block the EC3b rosetting ability of 4×10^9 human E. After 1 h at room temperature, the antibody- and PBS-treated samples were centrifuged at 10,000 *g* for 30 min to remove immune precipitates. Each of the H- and/or CR_1-depleted serum reagents were then added to pellets of 1×10^9 EC3bi (2.1×10^4 iC3b per E), and following resuspension of the EC3bi, the mixtures were incubated for 2 h at 37°C. After three washes with BDVEA, the EC3bi were analyzed for conversion to EC3d,g by tests for the uptake of ^{125}I-labeled anti-C3c and anti-C3g (6). To distinguish nonspecific shedding of uncleaved iC3b molecules from the EC3bi, an additional 1×10^9 EC3bi were incubated in 1.0 ml of DGVB for 2 h at 37°C. I-specific C3c release was determined from this control (6).

C Receptor Assays. C receptors were assayed by rosette formation with EC3 or C3-ms

(13). The buffer used for rosette assays is listed in Tables I–V. With some recently purchased lots of microspheres, nonspecific binding of BSA-ms was so high that these lots of microspheres could not be used for preparation of C3-ms.

Assays For C Receptor Specificity. C receptor specificity was determined by treatment of C receptor cells with either anti-C-receptor antibody, C3 fragments, or C3d-OR before assay of C-receptors (13). The $F(ab')_2$-anti-CR_1 was 8.2 mg/ml in PBS and 5 μl (41 μg) was sufficient to inhibit CR_1 activity completely on 4×10^5 monocytes or neutrophils. Anti-Mac-1 was used to inhibit CR_3 activity (21). Monocytes and neutrophils (4×10^5) were treated with 10 μl to 20 μl of anti-Mac-1 tissue culture supernatant and then washed once before assay of C receptors. The concentrations of C3 fragments used for inhibition are listed in Table II. 50 μl of C3d-OR was required to produce 50% inhibition of EC3d rosette formation with Raji cells, and the same amount of C3d-OR was used in experiments with monocytes and neutrophils.

In experiments that examined the divalent cation requirements for EC3bi binding to CR_3 and K, the EC3bi and monocytes (anti-CR_1-treated) were first washed with 20 mM EDTA/HBSS/BSA and then washed twice and resuspended in HBSS/BSA containing either 2 mM calcium chloride, a mixture of 2 mM EGTA and 2 mM magnesium chloride, or a mixture of 2 mM calcium chloride and 2 mM magnesium chloride.

N-acetyl-D-glucosamine (NADG) was purchased from BDH chemicals Ltd. (Poole, England) and various amounts were added to BDVA. After incubation of both EC3bi and monocytes (anti-CR_1-treated) in the NADG/BDVA for 15 min at 22°C, the EC3bi were mixed with the monocytes and analyzed for rosette formation.

Purification and Assay of K. K was isolated from bovine serum by EDTA elution from yeast cell walls (22). K was titrated in U-bottom microtiter plates for conglutination of EC3bi suspended at 1×10^8/ml in GVB containing either 2 mM calcium chloride, 2 mM each of EGTA and magnesium chloride, or 2 mM each of calcium chloride and magnesium chloride. Conglutination was allowed to develop for 2 h at 4°C before visual estimation of the 50% endpoint.

Results

Binding of B to C3b but Not to iC3b. Previous studies have demonstrated that under physiologic conditions B binds to C3b but not to iC3b, and that binding requires magnesium (Mg^{++}). Because the binding of B to C3b is charge-dependent (23) and enhanced by substitution of nickel (Ni^{++}) for Mg^{++} (25), studies were undertaken to determine if a binding site for B could be detected in iC3b using a low ionic strength buffer containing Ni^{++}. First, B was passed through two columns containing either ATS-C3b or ATS-iC3b in 1.0 mS buffer with Mg^{++}. All B was bound to the ATS-C3b and subsequently eluted with 10 mM EDTA in veronal-buffered saline. By contrast, B was not bound to ATS-iC3b in either Mg^{++} or Ni^{++}, and eluted in the same fractions as did hemopexin.

When B was assayed for binding to EC3b (4.5×10^4 C3b per E) by hemolytic assay in DGVB-Ni, 50% lysis was obtained with as little as 0.5 μg/ml of B (7.5 molecules B/molecule fixed C3b). However, when EC3bi (5.7×10^4 iC3b per E) were substituted for EC3b, no B-mediated hemolysis was detectable with B serial dilutions starting at 1.0 mg/ml (1.2×10^4 molecules B/molecule fixed iC3b).

Demonstration of a Binding Site for H and CR_1 in C3b, iC3b, and C3c. The binding of [^{125}I]-H to EC3b and EC3bi was examined in both low (4 mS) and isotonic salt buffers (Fig. 1). H binding to EC3b was readily detectable in GVB, whereas H binding to EC3bi was only observed in DGVB. The calculated affinity of H for EC3b was 5×10^6 M^{-1} in GVB and 1.8×10^7 M^{-1} in DGVB, and for

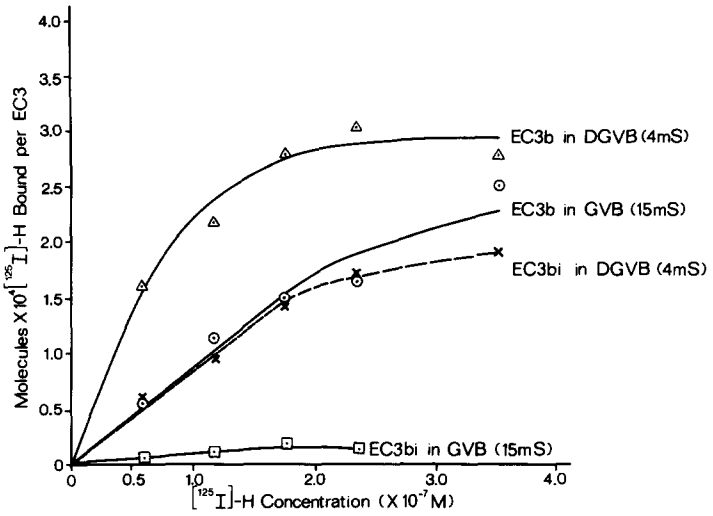

FIGURE 1. Binding of ^{125}I-labeled factor H to EC3b and EC3bi in isotonic versus low ionic strength buffer. Different amounts of [^{125}I]-H were added to either E, EC3b, or EC3bi in either GVB (15 mS at 22°C) or DGVB (4 mS at 22°C), and the amount of specifically bound [^{125}I]-H was determined by subtraction of the nonspecific [^{125}I]-H binding to E from the total binding of [^{125}I]-H to EC3b or EC3bi. The specific uptake of [^{125}I]-H in molecules per EC3b or EC3bi was plotted versus the initial unbound concentration of [^{125}I]-H. The EC3b were shown to bear 3.34×10^4 C3b molecules per cell by assay for uptake of ^{125}I-monoclonal anti-C3c, whereas the EC3bi were shown to bear 1.79×10^4 iC3b molecules per cell by assays for uptake of ^{125}I-anti-C3c and ^{125}I-anti-C3g (see Materials and Methods).

EC3bi in DGVB the affinity was 4×10^6 M^{-1}. The calculated number of H binding sites on EC3b and EC3bi was approximately the same as the number of bound C3b and iC3b molecules respectively as measured by the uptake of ^{125}I-monoclonal anti-C3c.

Because low ionic strength serum, but not isotonic serum, converted EC3bi to EC3d,g (6), it had been previously hypothesized that the H in low salt serum could serve as the I cofactor only because the low salt permitted H binding to EC3bi. To confirm this function of serum H, low ionic strength serum was treated with either anti-H, anti-CR_1, a mixture of the two antibodies, or PBS, and then examined for its ability to convert EC3bi to EC3d,g. Cleavage of EC3bi to EC3d,g was monitored by measuring the uptake of ^{125}I-monoclonal antibodies specific for C3c and C3g (6). As previously reported, C3c antigens were rapidly lost from EC3bi treated with 4 mS serum, whereas C3g antigens were retained, indicating conversion to EC3d,g. Previous addition to the 4 mS serum of anti-H, but not anti-CR_1 or PBS, inhibited C3c release by 85%, and a mixture of anti-H and anti-CR_1 inhibited C3c release by 94%.

The binding of iC3b and C3c to CR_1 was demonstrated with iC3b- and C3c-coated fluorescent microspheres (Table II) and with EC3bi (Table III). H binding to C3c was not examined. All rosette assays with C3-ms were performed in buffer containing EDTA that prevented the binding of iC3b to CR_3. Both iC3b-ms and C3c-ms were inhibited completely from binding to CR_1-bearing cells (erythrocytes, monocytes, neutrophils) by either anti-CR_1, or fluid-phase C3b, iC3b, or

TABLE II

Specificity Of Rosette Formation By Microspheres Coated With iC3b,C3d,g and C3c

Cell type	C3 fragment on micro-spheres	Rosette formation in the presence of						
		Buffer*	αCR_1‡	C3b§	iC3b	C3c	C3d,g	C3d-OR
		%	%	%	%	%	%	%
Erythrocytes	iC3b	75	0	0	0	11	73	69
	C3c	72	0	0	0	14	70	72
Monocytes	iC3b	96	0	6	0	21	97	92
	C3d,g	91	84	86	38	88	23	31
	C3c	80	0	0	0	12	76	77
Raji cells	iC3b	98	99	99	37	99	0	0
	C3d,g	99	99	99	51	99	3	0
	C3c	0	ND‖	ND	ND	ND	ND	ND

* Buffer: BDVEA.

‡ αCR_1: F(ab')$_2$-anti-CR_1, 41 μg/4 × 10^5 erythrocytes or leukocytes.

§ C3 fragment concentration: C3b, 500 μg/ml; iC3b, 400 μg/ml; C3c, 1.25 mg/ml; C3d,g, 350 μg/ml.

‖ ND, not done.

C3c (Table II). With Raji cells that express CR_2 (26) and lack CR_1 entirely (14, 27, 28), iC3b-ms binding was not inhibited by anti-CR_1, and C3c-ms rosettes were not observed. The results obtained with EC3bi were similar to those obtained with iC3b-ms; i.e. rosette formation in EDTA buffers was inhibited completely by anti-CR_1 (Table III). CR_1-dependent rosette formation with EC3bi required more C3 per cell than did EC3b rosette formation. With EC3b, erythrocyte rosette formation (immune adherence) was near maximum levels with 4 × 10^3 C3b per E (29, 30). However, with EC3bi, immune adherence was undetectable with 1.4 × 10^4 iC3b per E in isotonic salt buffer, and maximum levels of immune adherence required 3.5 × 10^4 iC3b per E and low-salt buffer (Table III). Even though EC3bi contained 5% C3b contamination, this C3b did not explain the CR_1 activity of EC3bi, as treatment of CR_1-positive EC3bi with 10 μg/ml of trypsin formed EC3d that still contained 3–5% contaminating C3b (Table I), but totally abolished binding to CR_1-bearing cells (E, monocytes, and neutrophils). When EC3bi rosette formation was assayed with phagocytes in buffers containing calcium and magnesium, EC3bi bound to both CR_1 and CR_3, so that little inhibition of rosette formation was produced by anti-CR_1 (Table III). By contrast, anti-CR_1 produced complete inhibition of human erythrocyte rosettes with EC3bi in calcium-magnesium buffer (BDVA), indicating that EC3bi did not bind to erythrocyte CR_3. With EC3bi bearing small amounts of iC3b (< 1.4 × 10^4 iC3b per E), rosette formation with monocytes and neutrophils in isotonic HBSS/BSA was primarily CR_3-dependent and selectively inhibited by anti-Mac-1 (Table III). As reported by Beller et al. (21), anti-Mac-1 had no effect on CR_1 activity measured either with EC3b (data not shown) or with EC3bi in EDTA (Table III).

Location of the CR_2-Binding Site in the d Region of iC3b, C3d,g, and C3d. Previous studies of CR_2 had demonstrated a specificity for both iC3b and C3d, and a

TABLE III

Differences in the C Receptor Specificity of EC3bi Rosette Formation Dependent Upon Divalent Cations, Ionic Strength, and Amount of iC3b

Cell type	Antibody treatment	C3bi mols per EC3bi ($\times 10^3$)	EC3bi Rosette Formation			
			HBSS/BSA (Ca, Mg, 14 mS)	EDTA/HBSS/BSA (14 mS)	BDVA (Ca, Mg, 4 mS)	BDVEA (EDTA 6 mS)
			%	%	%	%
Erythrocytes	—	14	0	0	21	10
	—	37	15	5	39	22
	—	65	22	8	60	32
	Anti-CR_1	65	0	0	0	0
Monocytes	—	7.9	59	0	86	0
	—	14	86	8	95	18
	Anti-CR_1	14	74	0	95	0
	Mac-1	14	5	6	20	13
	—	37	97	43	95	82
	—	65	96	77	97	85
	Anti-CR_1	65	85	0	94	0
	Mac-1	65	75	74	92	81
Neutrophils	—	14	96	2	100	11
	Anti-CR_1	14	79	0	100	0
	Mac-1	14	4	3	13	9
	—	37	99	78	100	84
	—	65	99	97	100	88
	Anti-CR_1	65	92	0	100	0
	Mac-1	65	95	93	100	86

complete absence of reactivity with C3b (13, 14, 26). Because earlier preparations of EC3bi and EC3d probably contained variable amounts of C3d,g, a C3 fragment that was previously unrecognized, fluorescent microspheres were coated with iC3b and C3d,g that were shown to be pure by SDS-PAGE, and then examined for binding to different cell types (Table II). In addition, the specificity of rosette formation with EC3bi (Table III) and EC3d,g (Table IV) was characterized.

Both iC3b-ms and C3d,g-ms rosettes with CR_2-bearing Raji cells were inhibited by fluid-phase iC3b, C3d,g, and C3d-OR (Table II). Similar results were obtained with EC3bi and EC3d,g (data not shown). Furthermore, C3d-ms binding to Raji cells was blocked by fluid phase iC3b and C3d,g (data not shown).

Monocytes and neutrophils that had been thought to lack CR_2 because of absence of rosetting ability with EC3d and C3d-ms (13), bound C3d,g-ms (Table II) and EC3d,g containing more than 5×10^4 C3d,g per E (Table IV). C3d,g-ms rosettes with phagocytic cells were not inhibited significantly by EDTA and/or anti-CR_1 (Table II), indicating that binding was probably not due to small amounts of contaminating C3b or iC3b binding to CR_1 and CR_3. Both C3d,g-ms and EC3d,g rosettes with phagocytes were inhibited by C3d-OR (Tables II and IV), suggesting the presence of a phagocyte CR_2-like C3d receptor. However, because the EC3d,g was known to contain small amounts of contaminating C3b

TABLE IV
Specificity of EC3d,g Rosette Formation

Cell type	EC3d,g* Rosette Formation in the presence of			
	Buffer‡	Anti-CR_1	C3d-OR	Anti-CR_1 + C3d-OR
	%	%	%	%
Erythroyctes	0	ND§	ND	ND
Monocytes	87	74	12	3
Neutrophils	95	76	48	21
Raji cells	99	99	46	48

* EC3d,g: 6.9×10^4 molecules of C3d,g per E, with C3b/iC3b contamination of 1.5×10^3 molecules per E determined by uptake of ^{125}I-anti-C3c.
‡ Buffer: BDVA.
§ ND, not done.

and iC3b, and because the C3d-OR contained 5% C3b-OR, a potent inhibitor of CR_1 (20), inhibition studies with anti-CR_1 were performed to determine if EC3d,g bound to CR_1 (Table IV). Amounts of anti-CR_1 sufficient to inhibit completely EC3b rosette formation, did produce ~18% inhibition of monocyte and neutrophil rosette formation with EC3d,g. Even though this was much less than the 72% inhibition (average) produced by C3d-OR, a mixture of anti-CR_1 and C3d-OR consistently produced more inhibition than did C3d-OR alone, suggesting that some phagocyte rosettes with EC3d,g were due to contaminating C3b or iC3b binding to CR_1. Since the EC3d from which the C3d-OR were prepared contained virtually no iC3b detectable with ^{125}I-anti-C3g, it appears unlikely that the C3d-OR contained iC3b-OR that inhibited EC3d,g from binding to CR_3. Furthermore, despite the finding that monocyte and neutrophil rosettes with EC3d,g were not observed in the presence of EDTA (data not shown), C3d,g-ms did bind to monocytes (Table II) and neutrophils (not shown) in the presence of EDTA and anti-CR_1.

The observation that monocytes and neutrophils expressed C3d-specific receptors that bound EC3d,g and C3d,g-ms, but not EC3d or C3d-ms (13), suggested the possibility that phagocytic cells might express low numbers of CR_2, and that these CR_2 were detectable only with EC3d,g and C3d,g-ms because C3d,g had a higher affinity for CR_2 than did C3d. Comparison of EC3d,g and EC3d CR_2-dependent rosette formation with Raji cells supported this hypothesis (Fig. 2). Raji cells bound EC3d,g that contained as few as 700 molecules of C3d,g per E, and nearly all cells were rosetted with EC3d,g bearing 2,200 C3d,g per cell. By contrast, Raji cells did not form rosettes with EC3d containing 900 C3d per E, and maximum rosette formation required ~5,000 C3d per E.

Because CR_2 had a higher affinity for C3d,g than for C3d, EC3d,g were coated with saturating amounts of monoclonal anti-C3g to determine if this blocked the CR_2-binding site. The observation that this treatment of EC3d,g (700 C3d,g per E) did not inhibit Raji cell rosettes, probably indicates that the g region does not form part of the CR_2-binding site.

Demonstration of a Common Binding Site for CR_3 and K in iC3b. Because iC3b bound to both CR_1 and CR_3 (Table III), the characteristics of CR_3 binding to iC3b could only be examined with granulocytes on which the CR_1 was blocked

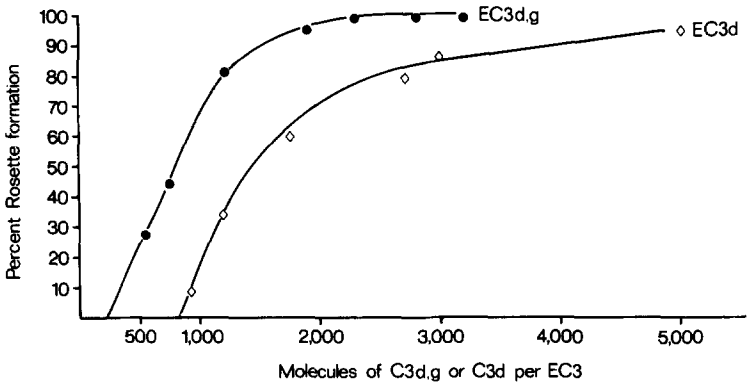

FIGURE 2. Raji cell rosette formation with EC3d,g and EC3d prepared with different amounts of C3. Different $EC3b_T$ were prepared with 1×10^{10} E and varying amounts of C3 (0.5 mg to 3.0 mg), 1% trypsin (wt/wt), and incubation for 1 min at 37°C. Each different $EC3b_T$ was divided into two parts that were converted into either EC3d,g or EC3d and then analyzed for uptake of ^{125}I-labeled monoclonal anti-C3c, anti-C3g, and anti-C3d. Rosette formation was carried out in BDVEA buffer by incubation for 15 min at 37°C on a tube rotator (see Materials and Methods).

TABLE V

Requirements for Divalent Cations and Carbohydrate in the Binding of EC3bi to CR_3 and Bovine Conglutinin

	Molecules of iC3b/ EC3bi	EC3bi rosettes with anti-CR_1-treated monocytes (CR_3)	EC3bi conglutination titre (K)*
		%	
BDVA	7.9×10^3	44	128
+ 3 mM NADG		36	0
+25 mM NADG		2	0
BDVA	1.4×10^4	84	6,400
+ 3 mM NADG		85	0
+ 25 mM NADG		57	0
HBSS/BSA‡	1.4×10^4		
+ 20 mM EDTA		0	0
+ 2 mM Mg-EGTA		28	0
+ 2 mM $CaCl_2$		71	6,400
+ 2 mM $MgCl_2$ and 2 mM $CaCl_2$		96	3,200

* One preparation of purified K, 100 μg/ml, was titrated under the different conditions listed.

‡ HBSS/BSA: Calcium and magnesium-free HBSS with 1% BSA.

with saturating amounts of anti-CR_1. CR_3 resembled K in that CR_3 bound only to iC3b and did not bind to C3b, C3c (13, 31), C3d,g (Table IV and reference 31) or C3d (13, 31). In addition, both CR_3 and K binding to EC3bi was inhibited completely by EDTA (Table III and reference 32). Because K binding to EC3bi was known to require both calcium cations and carbohydrate (32), CR_3 was compared to K for these two characteristics (Table V). Among the several sugars

known to inhibit K activity, *N*-acetyl-D-glucosamine (NADG) had been shown to be inhibitory at the lowest concentration, 3 mM (33). With EC3bi containing amounts of iC3b (7.9×10^3 molecules per E) that were suboptimal for CR_3-dependent rosette formation and K activity, 3 mM NADG produced little inhibition of rosette formation, but completely inhibited K activity. Higher amounts of NADG did produce significant inhibition of CR_3 activity in a dose-dependent manner, with nearly complete inhibition of CR_3 obtained with 25 mM NADG. However, with EC3bi containing greater amounts of iC3b (1.4×10^4 molecules per E), 25 mM NADG consistently produced an average of only 32% inhibition of CR_3 activity, while completely blocking conglutination.

To examine CR_3 and K cation requirements, EC3bi and monocytes in EDTA/HBSS/BSA were washed into HBSS/BSA containing either magnesium and EGTA (to chelate calcium), calcium only, or a mixture of magnesium and calcium, and then examined for CR_3 and K activity (Table V). Although both CR_3 and K activity were restored with calcium, CR_3 differed from K in that a low level of CR_3 activity was detectable in magnesium-EGTA, and calcium and magnesium produced much greater CR_3 activity than did calcium alone. In another type of test, CR_3 also resembled K in that both CR_3-dependent neutrophil EC3bi rosettes and clumps of conglutinated EC3bi were disrupted by addition of EDTA followed by mild agitation.

Discussion

The major conclusion of the present study was that C3 contained at least 4 distinct binding sites for 6 of 11 known C3-ligands. One binding site for factor B was present in C3b, but was destroyed completely by factor I cleavage of C3b into iC3b. A second binding site for factor H and CR_1 was expressed in the c region of C3b, and was still detectable, although with reduced affinity, in iC3b and C3c. A third binding site for CR_2 was located in the d region of C3 and was exposed only after I cleavage of C3b into iC3b or C3d,g. The data suggested that the very anionic g portion of C3d,g might enhance the charge-dependent binding affinity of CR_2 for C3d. The fourth binding site included a carbohydrate moiety of C3 that was exposed only in the iC3b fragment and bound both CR_3 and conglutinin (K).

C3 has a central role in both the classical and alternative pathways, and has more functions than any other single C component. Each function of C3 is dependent upon the expression within the molecule of a variety of different binding sites, as no enzymatic activity has ever been found in the molecule. After C3 activation, the labile binding site is generated in C3b by disruption of an internal thiolester bond in the d region (34), and this results in a proportion of the C3b molecules becoming bound covalently to nearby carbohydrate or protein surfaces. The C3a and C3b fragments and the products of C3b degradation by I (iC3b, C3c, and C3d,g) and trypsin (C3d) express binding sites for eleven different ligands: C3a receptor, B, properdin, H, C5, I, CR_1, CR_2, CR_3, C4-binding protein, and K. Many previous studies that used different techniques have demonstrated distinct binding sites for several pairs of these ligands. For example, the concurrent binding to C3b of both Bb and properdin (35), or of H and I (36), or of H and properdin (36), indicated that each member of the pair

must have a distinct binding site. More recently, the finding of monoclonal anti-C3 antibodies that would selectively inhibit either the B, H, or properdin binding sites, indicated separate binding sites for each of these ligands (37, 38).

The loss of hemolytic activity following H and I cleavage of C3b into iC3b has been assumed to indicate that I destroyed the B-binding site in C3b. However, it remained possible that a low affinity and/or hemolytically inactive B-binding site might be present in iC3b. To investigate a possible B-binding site in iC3b, both low ionic strength and nickel cations, each of which are known to enhance the binding of B to C3b (23, 25), were used with either ATS-iC3b or EC3bi. Because no binding of B to iC3b was detected, the B-binding site in C3b is probably destroyed totally by the initial two cleavages with I.

Previous reports of H and CR_1 binding to C3b suggested either a common binding site or partially overlapping sites. The binding of both H and CR_1 to C3b is enhanced by low salt buffer (18, 23, 39), and both ligands accelerate the dissociation of Bb from C3b,Bb,P and serve as cofactors for I cleavage of C3b and iC3b (6, 8, 10). In addition, high concentrations of H competitively inhibit CR_1 binding to EC3b (40). Even though H binding to EC3bi was not detected by past investigators (21), the finding that H could serve as an I cofactor for iC3b cleavage into C3c and C3d,g in low salt buffer (6), suggested that a low affinity H-binding site might be present in iC3b. Indeed, [^{125}I]-H binding to EC3bi was detectable only in 4 mS DGVB and not in 15 mS GVB (Fig. 1). As reported by Kazatchkine et al. (23), the H-binding affinity for EC3b was enhanced by low-salt buffer from 5×10^6 M^{-1} in GVB to 1.8×10^7 M^{-1} in DGVB. The H-binding affinity for EC3bi in DGVB (4×10^6 M^{-1}) was only slightly less than the H affinity for EC3b in isotonic buffer. The numbers of H-binding sites on EC3b and EC3bi were approximately the same as the numbers of C3b and iC3b molecules per cell respectively measured with ^{125}I-monoclonal anti-C3. This indicated that nearly all iC3b molecules bound H in low salt buffer, and that H-binding to EC3bi was not due to the small amount (<5%) of contaminating C3b on EC3bi.

The observed binding of H to EC3bi in low salt buffer appeared to confirm the mechanism by which low ionic strength permitted either purified H or serum H to be an I-cofactor for cleavage of fixed iC3b (6). However, because serum might contain very low amounts of free CR_1, and because the binding of CR_1 to EC3bi was also increased in low salt buffer (Table III), the possibility existed that undetected CR_1 in low ionic strength serum might be the factor I-cofactor for iC3b cleavage. Tests of low ionic strength serum however, demonstrated that H and not CR_1 was the primary I-cofactor for cleavage of fixed iC3b. Treatment of 4 mS serum with anti-H, but not anti-CR_1, inhibited C3c release from EC3bi by 85%. The possibility remains that a low concentration of free CR_1 in isotonic serum might serve as an I cofactor for the iC3b cleavage that has been observed in serum after C3 activation with cobra factor and incubation for 6–24 h at 37°C (17).

All previous reports have failed to demonstrate EC3bi binding to CR_1 (13, 18, 21, 41). The data presented in Tables II and III show the two major reasons for this. First, fixed iC3b can bind to all three types of C3 receptors. Second, the binding of EC3bi to human E is weak and requires both large amounts of fixed

iC3b and low ionic strength. With phagocytic cells, EC3bi binds to both CR_1 and CR_3, so that neither anti-CR_1 nor fluid-phase C3b produces much inhibition of EC3bi rosette formation (Table III and references 13, 41). Similarly, lymphocytes bind iC3b-ms and EC3bi to both CR_1 and CR_2 (Table II and reference 13). The binding of iC3b-ms and EC3bi to monocyte and neutrophil CR_1 was demonstrated by anti-CR_1 inhibition of rosettes in EDTA buffers that prevented iC3b binding to CR_3 (Table II and III). Anti-CR_1 also blocked EC3bi rosettes completely with human E that lacked CR_3 detectable by EC3bi rosette formation.

EC3bi bearing small amounts of iC3b bound primarily to CR_3 and not to CR_1. When these EC3bi were tested in isotonic buffers containing calcium and magnesium, monocyte and neutrophil rosette formation was unaffected by anti-CR_1 but almost completely inhibited by anti-Mac-1 (Table III, reference 21). With greater amounts of fixed iC3b, the EC3bi bound to both CR_1 and CR_3, and neither anti-CR_1 nor anti-Mac-1 produced significant inhibition of rosettes. An important recent finding provided additional confirmation of both the ability of phagocyte CR_1 to bind EC3bi and the identity of CR_3 with Mac-1. Two sibling patients were discovered who had an apparent genetic deficiency of the leukocyte Mac-1 antigen. Monocytes and neutrophils from these patients formed normal percentages of EC3bi rosettes in calcium-magnesium buffer, but these EC3bi rosettes were inhibited completely by $F(ab')_2$ anti-CR_1 (Ross, G. D., R. A. Thompson, M. J. Walport, R. H. R. Ward, J. Lida, R. A. Harrison, and P. J. Lachmann, manuscript in preparation).

iC3b had a much lower affinity for CR_1 than did either C3b or C4b. Maximum levels of immune adherence in low ionic strength buffer required ~9 times more iC3b than C3b (3.5×10^4 iC3b per EC3bi vs. 4.0×10^3 C3b per EC3b) and ~3 times more iC3b than C4b (9.0×10^3 C4b per EAC14b) (29, 30). Because EC3bi contained ~5% contaminating C3b, it appeared possible that EC3bi binding to CR_1 might be due entirely to this contaminating C3b. However, this was discounted because treatment of EC3bi with 10 μg/ml trypsin, that cleaved fixed iC3b to C3d but did not cleave contaminating C3b (Table I) (42), completely abolished detectable CR_1-dependent rosetting of the resulting EC3d. CR_1-dependent rosetting with C3b (18, 20), C4b (18), and iC3b (Table III) was considerably enhanced by low salt buffers, demonstrating that the CR_1-binding site was partially charge dependent.

The identification of a low affinity binding site(s) in iC3b for CR_1 and H supported previous findings that the CR_1-binding site was detectable in C3c (13, 30). In the present study, highly purified C3c was coupled to fluorescent microspheres, and the resulting C3c-ms were shown to bind only to CR_1. Attempts to use C3c-ms to investigate [^{125}I]-H binding to C3c were not successful, as a density of oil could not be found that would allow rapid separation by centrifugation of the C3c-ms from an aqueous phase containing unbound [^{125}I]-H.

The binding site for CR_2 had been previously shown to be present in both iC3b and C3d, but not in C3b (4, 13). The C3d,g fragment, that is probably the normal bound end product of C3 degradation (6, 17), had not been previously examined for binding to C receptors or other C3 ligands. Both C3d,g-ms (Table II) and EC3d,g (Table IV) bound to CR_2, as rosetting was inhibited by C3d-OR.

However, a comparison of EC3d,g and EC3d indicated that CR_2 had a higher affinity for C3d,g than for C3d (Fig. 2). Although fluid-phase C3d inhibited EC3d rosette formation (13), more than 1.0 mg/ml of C3d was required to inhibit EC3d,g rosette formation (data not shown). Because of this finding and the demonstration of C3d,g-ms (Table II) and EC3d,g (Table III) binding to phagocytic cells, it was at first believed that C3d,g bound to CR_3 (43, 44). Subsequently however, C3d-OR complexes were found to block C3d,g-ms and EC3d,g rosette formation with all cell types, and anti-Mac-1 (anti-CR_3) was found to have no effect on EC3d,g rosette formation (data not shown). C3d-OR resembled C3b-OR (20) in having a much greater ability to block C receptors than did uncomplexed C3d or C3b. The most potent inhibitor of CR_2 was C3d,g-OR. The CR_2 on Raji cells were completely blocked by treatment with microgram amounts of C3d,g-OR, and Raji cells were still unable to form EC3d,g or EC3d rosettes after three washing steps (Ross, G., unpublished observation). Because EC3d,g had a higher affinity for CR_2 than did EC3d, the g portion of EC3d,g was saturated with monoclonal anti-C3g to determine if this might block the CR_2-binding site. Because the attachment of an intact IgG molecule to the g region (8,000 daltons) caused no detectable inhibition of Raji cell rosette formation, it appears unlikely that C3g forms a part of the CR_2-binding site. An alternative explanation for the higher affinity of CR_2 for C3d,g as compared to C3d may be the much greater electrophoretic charge of C3d,g (17). As with CR_1 and CR_3, the rosetting activity of CR_2-bearing cells was greatly enhanced in low ionic strength buffer (26), indicating that charge interactions were very important for the CR_2-binding site. Perhaps the charge contributed by the anionic g region of C3d,g enhances the attraction of CR_2 for the d region of C3d,g.

The enhanced CR_2 binding of C3d,g as compared to C3d probably also explains the observed rosette formation of monocytes and neutrophils with C3d,g-ms and EC3d,g (Table II and IV). Because phagocytes did not bind C3d-ms (13) or EC3d (13, 21), nor stain with anti-CR_2 (26), these cells were previously believed to lack CR_2. EC3d,g binding to phagocytic cells required $>5.0 \times 10^4$ C3d,g molecules per EC3d,g, and was greatly enhanced by low ionic strength buffer. Because EC3d,g contained ~5% C3b and iC3b detectable with [^{125}I]-anti-C3c (Table I), the involvement of this contaminating C3b and iC3b in EC3d,g rosette formation was investigated. Anti-CR_1 did produce 18% inhibition of monocyte-EC3d,g rosettes, but C3d-OR produced an average of 72% inhibition. In addition, C3d,g-ms binding to phagocytes was inhibited by C3d-OR but not by anti-CR_1 in EDTA buffers, indicating that C3d,g-ms did not bind to CR_1 or CR_3 (Table II). Thus, the data suggest that phagocytic cells may express very small amounts of CR_2. In the future, tests for anti-CR_2 inhibition of phagocyte EC3d,g rosettes will be required to confirm this conclusion.

Because iC3b binds to all three types of C3 receptors, it has been particularly difficult to define the cell type distribution of CR_3. For example, iC3b-dependent rosette formation with anti-CR_2- or C3d-treated B lymphocytes was thought to detect B lymphocyte CR_3 (13). With recognition both that B lymphocyte CR_1 could bind to fixed iC3b and that B cells lacked the Mac-1 antigen (19), it now appears likely that B cells lack CR_3 and express only CR_1 and CR_2. On the other hand, the cytotoxic activity of antibody-dependent killer cells, that express the

Mac-1 antigen (19) and lack CR_1 (28), has been shown to be enhanced by target cell-bound iC3b (45), probably indicating that this other type of lymphoid cell does indeed express CR_3.

A particularly interesting finding was that CR_3 had a conglutininlike binding activity for fixed iC3b. Previous studies of EC3bi binding to phagocytic cells did not detect any significant inhibition by EDTA or NADG, as EC3bi were bound to CR_1 whenever CR_3 were inhibited. Characterization of the CR_3-binding site thus required prior blockade of neutrophil or monocyte CR_1 with anti-CR_1. Comparison of CR_3 and K revealed that both ligands required calcium and were inhibited by NADG (Table V). However, CR_3 differed from K in that a small amount of CR_3 activity was detected in magnesium/EGTA, and a mixture of calcium and magnesium produced significantly more CR_3 activity than did calcium alone. In addition, CR_3 activity was less sensitive to NADG than was K activity (Table V). Thus, while both CR_3 and K may bind to the same carbohydrate in iC3b, the CR_3 requirement for magnesium and greater resistance to NADG inhibition suggests that CR_3 may bind to a second site in iC3b that does not bind to K.

Additional experiments in the future will be required to confirm the identity of a single binding site for H and CR_1. It is of interest that a C3-affinity-purified antibody was produced that was specific for the idiotype of anti-H antibody and blocked the uptake of [^{3}H]-labeled H onto both EC3b and lymphocyte H receptors (46). This same anti-anti-H not only bound to EC3b (46), but also bound to EC3bi (Lambris, J., unpublished observation), and thus was probably specific for the H-binding site of C3. In the future, attempts will be made to produce more of this antibody to ascertain if it blocks simultaneously both the H and CR_1 binding sites in C3b. The similarity of the CR_3 and K binding sites also requires further study. K not only binds a carbohydrate in fixed iC3b, but also is known to bind directly to certain mannan-containing extracts derived from zymosan (42). Because monocytes express mannan-specific receptors (47), and neutrophils are able to bind weakly to unopsonized zymosan (48), it will be especially important to determine if phagocyte CR_3 are mannan-specific and able to bind yeast particles to phagocytes. Finally, the similarity of CR_3 and K suggests the possibility that bovine serum K may be a secreted form of leukocyte membrane CR_3.

Summary

The many different recognized functions of C3 are dependent upon the ability of the activated C3 molecule both to bind covalently to protein and carbohydrate surfaces and to provide binding sites for as many as eleven different proteins. The location of the binding sites for six of these different proteins (factors B and H, complement receptors CR_1, CR_2 and CR_3, and conglutinin) was examined in the naturally occurring C3-fragments generated by C3 activation (C3b) and degradation by Factor I (iC3b, C3c, C3d,g) and trypsin (C3d). Evidence was obtained for at least four distinct binding sites in C3 for these six different C3 ligands. One binding site for B was detectable only in C3b, whereas a second binding site for H and CR_1 was detectable in both C3b and iC3b. The affinity of the binding site for H and CR_1 was charge dependent and considerably

reduced in iC3b as compared to C3b. H binding to iC3b-coated sheep erythrocytes (EC3bi) was measurable only in low ionic strength buffer (4 mS). The finding that C3c-coated microspheres bound to CR_1, indicated that this second binding site was still intact in the C3c fragment. However, H binding to C3c was not examined. A third binding site in C3 for CR_2 was exposed in the d region by factor I cleavage of C3b into iC3b, and the activity of this site was unaffected by the further I cleavage of iC3b into C3d,g. Removal of the 8,000-dalton C3g fragment from C3d,g with trypsin forming C3d, resulted in reduced CR_2 activity. However, because saturating amounts of monoclonal anti-C3g did not block the CR_2-binding activity of EC3d,g, it appears unlikely that the g region of C3d,g or iC3b forms a part of the CR_2-binding site. In addition, detergent-solubilized EC3d (C3d-OR) inhibited the CR_2-binding activity of EC3d,g. Monocytes and neutrophils, that had been previously thought to lack CR_2 because of their inability to form EC3d rosettes, did bind EC3d,g containing $>5 \times 10^4$ C3d,g molecules per E. The finding that monocyte and neutrophil rosettes with EC3d,g were inhibited by C3d-OR, suggested that these phagocytic cells might indeed express very low numbers of CR_2, and that these CR_2 were detectable with EC3d,g and not with EC3d because C3d,g had a higher affinity for CR_2 than did C3d. A fourth C3 binding site for CR_3 and conglutinin (K) was restricted to the iC3b fragment. Because of simultaneous attachment of iC3b to phagocyte CR_1 and CR_3, the characteristics of iC3b binding to CR_3 could only be examined with phagocytes on which the CR_1 had been blocked with anti-CR_1. Inhibition studies with EDTA and *N*-acetyl-D-glucosamine demonstrated a requirement for both calcium cations and carbohydrate in the binding of EC3bi to CR_3 and to K. However, CR_3 differed from K in that magnesium cations were required in addition to calcium for maximum CR_3 binding activity, and NADG produced less inhibition of CR_3 activity than of K activity.

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