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Structural and functional analysis of CR2/EBV receptor by means of monoclonal antibodies and limited tryptic digestion

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SUMMARY

The receptor for the C3d fragment of the third component of complement, CR2, has recently been shown also to act as the receptor for the Epstein–Barr virus (EBV) and to be involved in the control of B-cell proliferation. In order to define functionally important domains on this molecule, we produced monoclonal antibodies to several distinct epitopes. CR2 was purified from a NP-40 lysate of human tonsils by a new method involving sequential chromatography on lentil lectin Sepharose 4B and DEAE–Sephadex and used to immunize mice. After fusion we obtained four stable hybridoma lines producing antibody to CR2. Specificity of these antibodies for CR2 was ascertained by immunofluorescence analysis on a panel of various cells known to possess CR2, by their reactivity in a recently described ELISA for C3 receptors, by Western blotting with purified CR2 and immunoprecipitation from ^{125}I-labelled Raji cells. These four antibodies were found to recognize three distinct epitopes localized on the same fragments of 95,000, 72,000, 50,000, 32,000 and 28,000 MW obtained after mild tryptic digestion of CR2. The 72,000 MW fragment contains the binding site for C3d. Two monoclonal antibodies recognizing the same epitope did not inhibit the binding of C3d-coated sheep erythrocytes to Raji cells, whereas the other two antibodies against distinct epitopes did inhibit in the presence of a second antibody. All four monoclonal antibodies stimulated the proliferation of human peripheral blood B cells.

INTRODUCTION

The receptor for the C3d fragment of the third complement component, CR2, has been shown to be a glycoprotein of 145,000 MW (Barel, Charriaut & Frade, 1981; Weis, Tedder & Fearon, 1984; Iida, Nadler & Nussenzweig, 1983; Micklem, Sim & Sim, 1984) expressed on human B cells (Tedder, Clement & Cooper, 1984), human follicular dendritic cells, human pharyngeal epithelial cells (Young *et al.*, 1986), cervical epithelium (Sixbey, Lemon & Pagano, 1986) and HTLV-I-transformed human T-cell lines (Schulz *et al.*, 1986a). It has strong affinity for the C3d and C3dg fragments of the third complement component C3, but also binds with lower affinity to C3bi (Ross & Medof, 1985). CR2 also serves as the attachment site of Epstein–Barr virus (EBV) on B cells (Frade *et al.*, 1985a; Fingeroth *et al.*, 1984; Nemerow *et al.*, 1985), and we as well as other groups have recently shown that CR2 plays a role in the control of B-cell proliferation (Nemerow, McNaughton & Cooper, 1985; Frade *et al.*, 1985b; Melchers *et al.*, 1985). Cross-linked C3 replaces the action of macrophage-derived growth factors controlling the entry of B cells into their S phase (Melchers *et al.*, 1985). B-lymphoblastoid cell lines shed a CR2-fragment of 72,000 MW into their culture supernatant that contains the binding site on CR2 for C3d since it can be purified by affinity chromatography on C3d–Sepharose (Lambris, Dobson & Ross, 1981; Micklem, Sim & Sim, 1985; Myones & Ross, 1987). In this study we produced four new monoclonal antibodies to CR2 and examined their capacity to inhibit the binding of C3d-coated sheep erythrocytes to B cells as well as to induce B-cell proliferation. In addition we mapped the epitopes recognized by these antibodies on tryptic and naturally occurring fragments of CR2.

Abbreviations: BCGF, B-cell growth factor; BSA, bovine serum albumin; C1, C2, C3, C4, first, second, third and fourth complement component, respectively; C3b, C3bi, C3dg, C3d, fragments of the third complement component, C3; iC3, haemolytically inactive C3; C4BP, C4-binding protein; CR1, C3b receptor; CR2, C3d/EBV receptor; DEAE, diethylaminoethyl; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HTLV-I, human T-lymphotropic virus I; NP-40, Nonidet P40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate.

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MATERIALS AND METHODS

Purification of CR2

Partial purification by lentil lectin and DEAE chromatography. A single cell suspension was prepared from human

tonsils (a kind gift from Innsbruck University Hospital), and the cells were lysed (5×10^7 cells/ml) in 10 mM Na-phosphate buffer, pH 7·2, containing 140 mM NaCl, 1% NP-40 (BDH Chemicals Ltd), 2 mM phenyl-methansulphonylfluoride (PMSF, Merck), 1 mM di-isopropylfluorophosphate (DFP, Sigma) for 30 min on ice. After centrifugation at 1000 *g* for 15 min to remove nuclei, the lysate was stored at −70° until used. One thousand millilitres of lysate were run over a 20-ml lentil lectin Sepharose 4 B column (Pharmacia) equilibrated in 25 mM Na-phosphate buffer, pH 7·4, containing 0·7 mM $MgCl_2$, 0·7 mM $CaCl_2$, 1 mM PMSF and 0·5% NP-40. After rinsing the column with 200 ml equilibration buffer followed by 200 ml of equilibration buffer containing 300 mM NaCl, bound proteins were eluted with 400 ml equilibration buffer containing 500 mM α-methyl-D-mannopyranoside (Serva). Fractions containing CR1 and CR2 were identified using a recently described ELISA for C3 receptors (see below, and Schulz *et al.*, 1985) in combination with monoclonal antibody 3F11 to CR1 (Schulz *et al.*, 1985) and anti-B2 (Nadler *et al.*, 1981) (Coulter) to CR2. These fractions were pooled, dialysed against 10 mM Na-phosphate, pH 7·4, containing 0·5% NP-40, 0·25 mM PMSF and applied to a 40 ml DEAE–Sephadex column (Pharmacia) equilibrated in the same buffer. After rinsing the column with 200 ml equilibration buffer, CR1 and CR2 were eluted with a linear gradient of 0–0·3 M NaCl (2 × 150 ml). Fractions containing CR1 and CR2 were identified as above and analysed on silver-stained SDS–polyacrylamide gels (Laemmli, 1970; Merril *et al.*, 1981).

Purification of CR2 by affinity chromatography. Monoclonal antibodies to CR2, 1C8 and 6F7 were purified from mouse ascites by precipitation with 40% NH_4SO_4, pooled and coupled to cyanogen bromide-activated Sepharose at 2 mg/ml Sepharose according to the manufacturer's instructions. Fifty millilitres of a detergent lysate of human tonsil cells prepared as above were incubated with 2 ml of monoclonal anti-CR2 Sepharose for 2 hr at room temperature, following which the Sepharose was poured into a disposable plastic column and washed with 60 ml of 10 mM phosphate buffer containing 150 mM NaCl, 1% NP-40, 0·01% SDS, with 20 ml of the same buffer containing 300 mM NaCl and finally with 20 ml 20 mM Tris-HCl, pH 7·5, and 0·01% SDS. Bound proteins were then eluted with 15 mM triethanolamine, pH 12, fractions of 700 μl were collected and immediately neutralized with 0·2 M glycine, pH 2·3. CR2 content and purity were again monitored by ELISA (Schulz *et al.*, 1985) and SDS–PAGE.

Purification of the 72,000 MW fragment of CR2 from Raji cell supernatant. This was performed as published elsewhere (Myones & Ross, 1987).

Iodination of purified CR2

Four micrograms affinity-purified CR2 were labelled to a specific activity of $3·6 \times 10^7$ c.p.m./μg by the iodogen method (Fraker & Speck, 1978) using 1·5-ml plastic tubes precoated with 30 μg of iodogen (Pierce Chemicals).

Trypsin digestion of labelled CR2

Five microlitres of labelled CR2 (equivalent to $1·8 \times 10^4$ c.p.m.) were incubated for 4 min at 37° with 100 ng or 250 ng of trypsin in 25 μl of PBS containing 0·01% BSA. The reaction was stopped with 10 μl 50 mM PMSF.

Monoclonal antibodies

Female BALB/c mice were immunized three times with 10 μg of CR2 purified to 60% purity by lentil lectin and anion exchange chromatography. Three days after the last booster, spleens were removed and fused to the Ag8.653 myeloma line as previously described (Fazekas de St Groth & Scheidegger, 1980). Supernatants were tested for the presence of antibodies to C3 receptors using a previously described ELISA for C3 receptors (Schulz *et al.*, 1985). Briefly, ELISA plates were coated with iC3 in 0·1 M $NaHCO_3$ buffer (20 μg/ml, 50 μl/well) and saturated with 1% ovalbumin (Sigma) in 10 mM K-phosphate buffer containing 50 mM NaCl. An NP-40 extract of human tonsil cells in the same buffer was added as a source of CR2. After washing away unbound material, hybridoma supernatants diluted in saturation buffer were added for 1 hr, followed by peroxidase-coupled rabbit anti-mouse Ig and azino-3-ethyl-benzthiazolin-sulphonic acid (15 mg/ml) in 10 mM K-phosphate buffer. Hybridomas secreting appropriate antibodies were cloned twice by limiting dilution. Antibodies were purified from mouse ascites by precipitation with 40% NH_4SO_4 and ion exchange chromatography on DEAE. Purified antibodies were labelled with *N*-hydroxy-succinimidobiotin as previously described (Guesdon, Ternynck & Avrameas, 1979).

Mapping of antibody epitopes on CR2

Fifty microlitres of a 1 : 1000 dilution (saturating concentration) of monoclonal antibody purified from mouse ascites by NH_4SO_4 precipitation were coated onto ELISA plates in the concentrations indicated in the text. After saturation of the plates with 1% BSA in PBS, an NP-40 lysate of tonsil cells was added for 30 min as a source of CR2. Plates were washed in PBS containing 0·05% Tween 20 and then incubated for 30 min with 20 μl of a biotinylated monoclonal antibody to CR2. After washing the plates, the amount of bound antibody was assessed by adding avidin-conjugated horseradish peroxidase (Sigma) and substrate as above.

Rosette inhibition assay

Sheep erythrocytes coated with amboceptor, C1, C4, C2 and C3d (EAC1423d) were prepared as previously described (Schmitt *et al.*, 1981). Thirty microlitres of lymphoblastoid cells (2×10^6/ml) were preincubated with 25 μl of monoclonal antibodies in different dilutions or buffer for 30 min at 37°. Thirty microlitres of EAC1423d ($1·3 \times 10^8$/ml) were then added for 30 min at 37° and rosette formation then evaluated. In some experiments a rabbit anti-mouse Ig antibody was added before adding the EAC1423d to cross-link the receptor molecules on Raji cells.

Stimulation of human B cells with monoclonal antibodies

Human tonsil cells were isolated by disrupting human tonsils with forceps and passing the cells through a metal net. Mononuclear cells were then isolated by centrifugation on Ficoll–Paque. T cells were removed by two cycles of rosetting with S- (2-amino-ethyl)-isothiuronium bromide-treated sheep erythrocytes followed by centrifugation on a Ficoll cushion. The resulting cell preparation was then depleted of monocytes by overnight culture on plastic petri-dishes in RPMI containing 10% FCS. This resulted in a preparation of cells that was highly enriched in B cells (90% CD19-positive cells). B cells were then cultured in triplicate at 5×10^4 cells/well in flat-bottomed

microtitre plates in RPMI containing 10% heat-inactivated fetal calf serum. Antibodies to CR2 and antibody 74 (directed against a serum protein of 220,000 MW), which was used as a control, were purified from mouse ascites by repeated Na_2SO_4 precipitation and added in the final concentrations indicated in the text. To some wells a T-cell derived growth factor was added that had been purified from the supernatants of phytohaemagglutinin A-stimulated T cells essentially as described by Frade *et al.* (1985b). In this publication it is referred to as 'BCGF-like growth factor' by virtue of its capacity to stimulate the growth of purified human tonsillar B cells in the presence but not in the absence of an antibody to the surface IgM of B cells. Because of the method of purification employed (NH_4SO_4 precipitation, chromatography on AcA 54), this preparation may still contain small amounts of other growth factors, i.e. IL-2. After 3 days of culture the plates were pulsed with 0·5 μCi of [^{3}H]thymidine/well for 12 hr, and then the cells were harvested and counted in a liquid scintillation counter.

Immunoprecipitation of trypsin-treated CR2 with monoclonal antibodies

CR2 fragmented with trypsin as described above (total volume of 40 μl) was incubated with 10 μl of Sepharose-bound anti-CR2 monoclonal antibody (2 mg/ml Sepharose) or anti-human μ-chain monoclonal antibody (clone 89) for 1 hr at room temperature. Sepharose beads were then washed three times in 10 mM phosphate buffer, pH 7·3, containing 150 mM NaCl, 0·5 mM EDTA, 0·01% SDS, 0·1% BSA, once in the same phosphate buffer containing 1% NP-40, 450 mM NaCl, and once in 50 mM Tris-HCl, pH 7·3. Bound proteins were eluted by boiling the Sepharose in 10 μl of reducing sample buffer (50 mM Tris, pH 6·8, 2% SDS, 10% glycerol, 1% mercaptoethanol) and electrophoresed on a SDS–polyacrylamide gel. The Coomassie-stained gel was dried and exposed to a Kodak X-omat film for 1–2 days at −80°. In some experiments trypsin-treated CR2 was reduced with dithiothreitol (final concentration 3 mg/ml), alkylated with iodoacetamide (final concentration 16 mg/ml) and dialysed against PBS containing 0·1% NP-40, 0·5 mM EDTA before immunoprecipitation with anti-CR2 monoclonal antibodies.

Immunoprecipitation of the 72,000 MW fragment purified from Raji cell supernatant was performed by the same method.

RESULTS

Partial purification of CR2 by lentil lectin and ion exchange chromatography

CR2 was partially purified from a detergent lysate of human tonsils as described in the Materials and Methods. Fractions containing CR2 and CR1 were identified using a recently described ELISA (Schulz *et al.*, 1985) for human C3 receptors and analysed on a silver-stained SDS–polyacrylamide gel (Fig. 1). The identity of the bands designated as CR1 and CR2 in Fig. 1 was verified by Western blotting with antibodies to CR1 [3F11 (Schulz *et al.*, 1985)] and CR2 [B2 (Nadler *et al.*, 1981)] (not shown). The resulting preparation of CR2 was about 60% pure with CR1 as the main contaminant. As illustrated in Fig. 1, CR2 elutes a little earlier than CR1 but cannot be clearly separated from CR1 by this method.

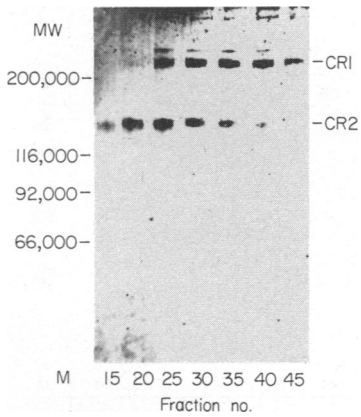

Figure 1. Purification of CR2 on DEAE-ion exchange chromatography: 50 μl of each DEAE–Sephadex fraction found to contain CR2 by ELISA (see the Materials and Methods) were run under reducing conditions on a 5–15% polyacrylamide gel, which was then silver-stained.

Table 1. Pattern of reactivity of monoclonal antibodies with various cells

	Receptor status		Binding to cells			
Cell	CR1	CR2	1C8	1F8	2G7	6F7
Tonsil cells*	+	+	+	+	+	+
Raji*	−	+	+	+	+	+
NC-37*	+	+	+	+	+	+
Jurkat*	−	−	−	−	−	−
Erythrocytes†	+	−	−	−	−	−

5×10^5 cells were incubated in 50 μl of monoclonal antibody supernatant for 30 min at 4°, followed by FITC-labelled anti-mouse Ig. The plus sign denotes more than 5% positive cells; in general, 70–90% of Raji cells, 50–60% of NC-37 and 40–60% of tonsil cells were positive with all monoclonal antibodies. For agglutination, 20 μl of human erythrocytes (1×10^8/ml in veronal buffer) were mixed with 20 μl of a serial dilution of monoclonal antibody supernatant followed after 30 min by 20 μl of rabbit anti-mouse Ig (1:1000, Dakopatts, Copenhagen, Denmark) in 96-well microtitre plates. Agglutination was read after 1 hr at room temperature. A monoclonal antibody to CR1 (3F11) served as a positive control (not shown).

* Tested in immunofluorescence.

† Tested in agglutination.

Production of monoclonal antibodies to CR2

Four hybridoma cell lines producing monoclonal antibodies to CR2, termed 1C8, 1F8, 2G7 and 6F7, were obtained. All of them recognized CR2 bound to iC3 that had been immobilized in an ELISA plate as described in the Materials and Methods. All antibodies reacted with cells or cell lines known to possess CR2 (Raji, NC 37, tonsil cells) but not with the T-cell line JURKAT or human erythrocytes, both negative for CR2 (Table 1). All four monoclonal antibodies reacted with a band of 145,000 MW on Western blots using the CR2 preparation described above as antigen (Fig. 2), confirming that they do in fact recognize CR2.

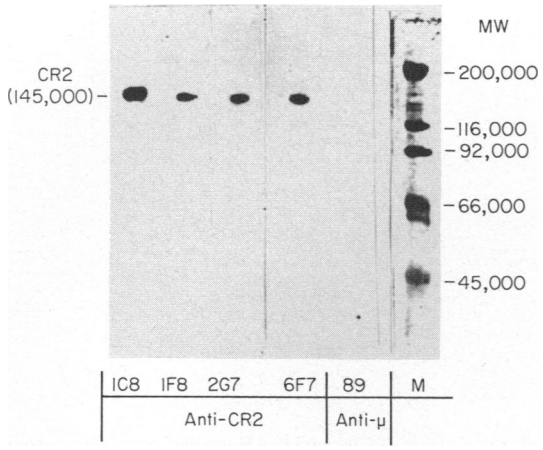

Figure 2. Reactivity of 1C8, 1F8, 2G7 and 6F7 with CR2: 50 μl of fraction 25 (see Fig. 1) were separated on a 5–15% SDS–polyacrylamide gel under non-reducing conditions, blotted to nitrocellulose and blots then stained with antibodies 1C8, 1F8, 2G7 and 6F7 followed by peroxidase-conjugated goat anti-mouse Ig and diaminobenzidine as previously described (Towbin, Staehelin & Gordon, 1979). Clone 89, directed at the human IgM heavy chain, served as a control.

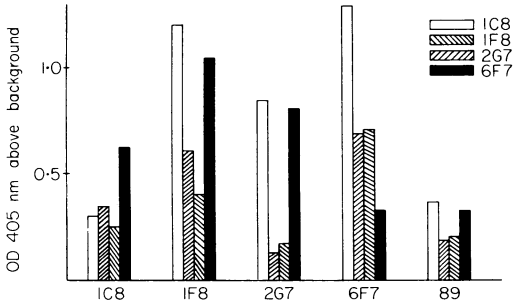

Figure 3. Binding to three distinct epitopes of anti-CR2 monoclonal antibodies: unlabelled monoclonal antibody to CR2 and human μ-chain (clone 89) as a control coated onto an ELISA plate as described in the Materials and Methods was used to trap CR2 from a NP-40 lysate of tonsil cells. The binding of the four biotinylated monoclonal antibodies to this complex was then measured by using peroxydase-conjugated avidin and ABTS as substrate (see the Materials and Methods). Monoclonal antibodies denoted on the abscissa represent the antibodies bound to the ELISA plate. The ordinate represents ELISA values after subtracting the values for the buffer control.

Localization of epitopes recognized by anti-CR2 antibodies

In a first series of experiments, we tested whether these four monoclonal antibodies to CR2 would react with identical or distinct epitopes. A given monoclonal antibody was attached to an ELISA plate and CR2 bound to this antibody. Then the plate was incubated with all four monoclonal antibodies in their biotinylated form followed by avidin-conjugated peroxydase and substrate. As shown in Fig. 3, CR2 bound to any one of these four antibodies is no longer recognized by the same antibody. However, CR2 bound to 1C8 is still recognized by 6F7, and CR2 bound to 6F7 is still recognized by 1C8, suggesting that the epitope recognized by 1C8 is distinct from the one defined by 6F7. Likewise, 6F7 and 1C8 both bind to CR2 trapped by 2G7 and 1F8, indicating that they react with epitopes distinct from the ones defined by 1F8 and 2G7. In contrast, 1F8 does not recognize CR2 bound to 2G7 and vice versa, indicating that these two monoclonal antibodies bind to the same or at least very closely situated epitopes.

We then tried to localize the epitopes defined by these four antibodies on proteolytic fragments of CR2. CR2 affinity-purified on 1C8 Sepharose was radiolabelled with ^{125}I and digested with limiting amounts of trypsin. The resulting fragments were then immunoprecipitated with 1C8, 1F8, 2G7, 6F7 and a monoclonal antibody to the μ-chain of human IgM (clone 89). As shown in Fig. 4, all four monoclonal antibodies bind proteolytic fragments with similar molecular weights of 95,000, 75,000, 50,000, 32,000 and 28,000 MW.

An 83,000 MW fragment of CR2 produced by trypsin digestion has recently been shown to carry the binding site for C3d (Micklem *et al.*, 1985). A CR2 fragment of similar molecular weight (72,000 MW) also carrying the binding site for C3d is found in the supernatant of B-lymphoblastoid cell lines (Lambris, Dobson & Ross, 1981; Micklem *et al.*, 1985; Myones & Ross, 1987). All four monoclonal antibodies to CR2 react with the 72,000 MW C3d-binding CR2 fragment from Raji cell supernatant (Fig. 5). The 72,000 MW fragment produced by trypsin cleavage that is recognized by all four monoclonal antibodies (Fig. 4) also binds to C3d (not shown).

Functional characteristics of anti-CR2 antibodies

In rosette inhibition experiments using sheep erythrocytes coated with amboceptor, C1, C4, C2, C3d and the Raji lymphoblastoid cell line, none of the monoclonal antibodies to CR2 inhibited rosette formation by itself. However, when a rabbit anti-mouse Ig was added in addition to the monoclonal antibody in order to cross-link CR2 molecules on the Raji cell surface, 1C8 and 6F7 did inhibit rosette formation whereas the two antibodies 1F8 and 2G7 recognizing the same or closely spaced epitopes did not inhibit (Table 2).

Since CR2 has been implicated in the control of B-cell proliferation (Melchers *et al.*, 1985; Nemerow *et al.*, 1985; Frade *et al.*, 1985b; Erdei *et al.*, 1985), and monoclonal antibodies to CR2 have been reported to differ in their capacity to stimulate human B cells (Nemerow *et al.*, 1985), we tested the effect of our antibodies on human B cells. As shown in Fig. 6, all antibodies were capable of inducing proliferation of human B cells in the presence of a T-cell derived growth factor. In the absence of this 'BCGF-like' factor, antibodies to CR2 had no effect.

DISCUSSION

The C3d/EBV receptor, CR2 or CD21, is one of the membrane molecules of B cells that are involved in transmitting growth-promoting signals to the interior of the B cell (Nemerow *et al.*, 1985; Frade *et al.*, 1985b; Melchers *et al.*, 1985). We report here a new procedure for the purification of CR2 from human tonsils by lentil lectin and ion exchange chromatography and the production of four monoclonal antibodies, the epitopes of which were mapped with respect to the C3d-binding fragment of CR2.

The purification procedure that we used to isolate sufficiently pure amounts of CR2 necessary for the immunization of

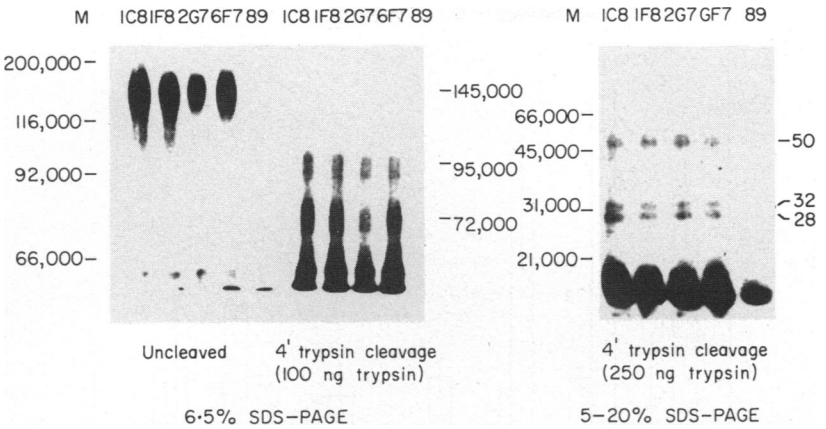

Figure 4. Binding of tryptic fragments of CR2 to anti-CR2 monoclonal antibodies: ^{125}I-labelled affinity-purified CR2 was incubated with 100 ng or 250 ng trypsin for 4 min as described in the Materials and Methods. Identical digests were then immunoprecipitated with either 1C8, 1F8, 2G7, 6F7 or clone 89 (anti-human μ-chain) and immunoprecipitates electrophoresed on a 6·5% or 5–20% SDS–polyacrylamide gel under reducing conditions. The dried gel was exposed to a Kodak X-Omat film for 2 days. Numbers on the right or left denote the position of molecular weights.

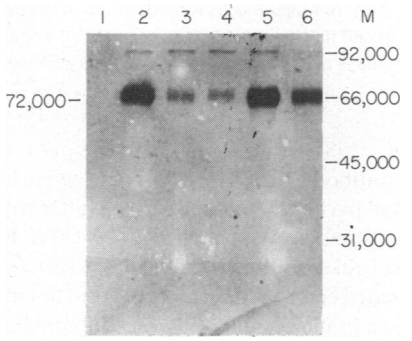

Figure 5. Binding of anti-CR2 monoclonal antibodies to the 72,000 MW fragment of CR2 purified from Raji-cell supernatant: the 72,000 MW fragment of CR2 purified from Raji-cell supernatant as previously described (Myones & Ross, 1987) was immunoprecipitated with 25 μl of Sepharose CL6B to which the following antibodies had been coupled: MAH-3, directed against factor H (Schulz *et al.*, 1984) (Lane 1), 1C8 (Lane 2), 6F7 (Lane 3), 2G7 (Lane 4), 1F8 (Lane 5), and OKB7 (Nemerow *et al.*, 1985) (Lane 6). Proteins bound to these Sepharoses were eluted with 15 mM triethanolamine, pH 12, as described in the Materials and Methods, separated on a 12% SDS–polyacrylamide gel and transferred to nitrocellulose. Blots were then stained with a pool of 1C8, 1F8 and OKB7 followed by peroxydase-conjugated anti-mouse Ig and substrate.

Table 2. Capacity of MABs to block EAC3D rosette formation with Raji

	% rosette formation in the presence of antibody diluted:				Rosette inhibition
	1:400	1:1600	1:6400	1:25,600	
(a) Without additional anti-mouse Ig					
1C8	93	82	88	87	–
1F8	83	88	87	91	–
2G7	96	90	80	80	–
6F7	93	82	78	93	–
PBS–BSA	90				–
(b) With additional anti-mouse Ig					
1C8	10	16	16	58	+
1F8	65	88	80	90	–
2G7	88	82	66	80	–
6F7	36	40	30	70	+
PBS–BSA	87				–

Rosette inhibition was performed as described in the Materials and Methods.

mice is a modification of a previously reported method for the purification of CR1 from human erythrocytes (Fearon, 1980). The fact that similar methods can be used for the isolation of CR1 as well as CR2 and the observation that CR1 and CR2 elute from the DEAE–sephadex column in neighbouring fractions (see Fig. 1) reflect the close biochemical relationship of these two membrane molecules. In fact, CR1 and CR2 seem to manifest a marked sequence homology (Weis *et al.*, 1986) and are both members of a family of structurally highly related complement proteins which include C4BP (Chung, Bentley & Reid, 1985) and factor H (Schulz *et al.*, 1986b), and are encoded near to each other on the short arm of chromosome 1 in humans (Weis *et al.*, 1987).

We obtained four hybridoma cell lines producing monoclonal antibody to CR2. By employing the C3 receptor ELISA (Schulz *et al.*, 1985) to screen for antibodies that would react with a protein from tonsil cells that had itself been bound to iC3, we identified only antibodies to CR1 and CR2. The specificity for CR2 of the monoclonal antibodies reported here was ascertained by establishing that they only react with cells known to possess CR2 but not CR1 (which was the main contaminant in the CR2 preparations used for immunization—see Fig. 1). In addition, all four monoclonal antibodies bound to a 145,000 MW band, corresponding to the molecular weight for CR2 (Fig. 2).

These four monoclonal antibodies define at least three distinct epitopes as shown in Fig. 3. The epitopes recognized by 1F8 and 2G7 may be identical or at least very close since neither

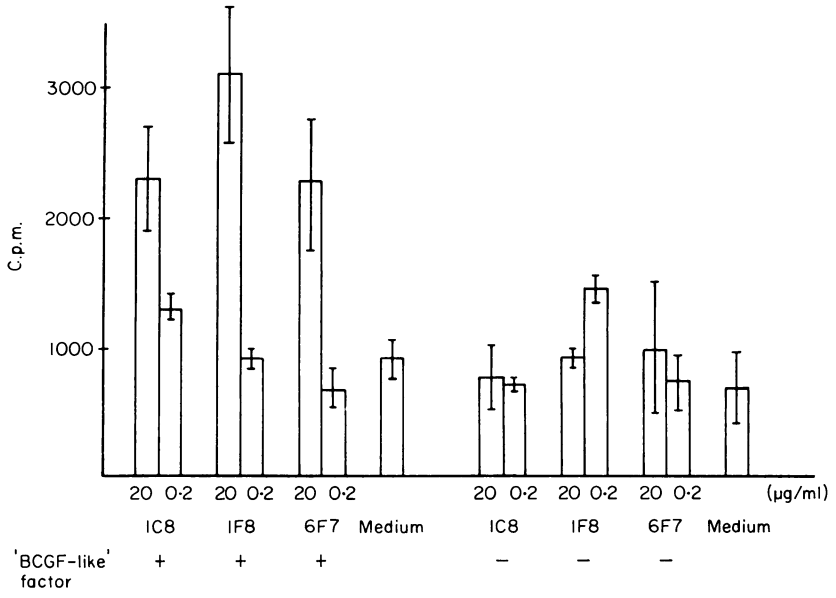

Figure 6. Induction of B-cell proliferation by monoclonal antibodies to human CR2: human tonsil B cells were cultured in the presence of purified monoclonal antibodies to CR2 (20 μg/ml and 0·2 μg/ml final concentration) and in the simultaneous presence (+) or absence (–) of a T-cell derived purified 'BCGF-like' factor (final concentration 15% v/v) for 3 days as described in the Materials and Methods.

of them binds well to CR2 trapped by the other. The fact that 2G7 and 1F8 do not bind well to CR2 trapped by 1C8 (Fig. 3, left column) is probably due to steric inhibition because in the reverse experiment 1C8 binds well to CR2 attached to 1F8 or 2G7. Similar results were obtained by blocking studies when unlabelled monoclonal antibody was used to inhibit the binding of biotinylated antibody to CR2 (not shown). Here again 1F8 and 2G7 seemed to compete for a binding site, whereas the other monoclonal antibodies did not block each other or 2G7 and 1F8.

The epitopes recognized by the four monoclonal antibodies could be localized on the 72,000 MW fragment of CR2, which is known to contain the binding site for C3d and which can be purified from the supernatant of Raji B-lymphoblastoid cells (Lambris *et al.*, 1981; Myones & Ross, 1987) (see Fig. 5). The 72,000 MW tryptic fragment precipitated by our four monoclonal antibodies (see Fig. 4) also binds to C3-sepharose (not shown) and is probably identical to the 83,000 MW tryptic fragment of CR2 reported by Micklem *et al.* (1985), because the CR2 fragment purified from Raji cell supernatants also ran at 83,000 in these authors' hands. Among previously described monoclonal antibodies to CR2, OKB7 also binds to this fragment of CR2 whereas B2 does not (Myones & Ross, 1987). 1C8, 2G7, 1F8 and 6F7 all precipitate smaller trypsin fragments of CR2 of about 50,000, 32,000 and 28,000 MW (Fig. 4). This indicates that the epitopes for these four monoclonal antibodies are localized on a 28,000 MW fragment of CR2. This finding is reminiscent of similar observations that we made in the case of complement factor H, where six monoclonal antibodies all bound to a 38,000 MW tryptic fragment (Alsenz *et al.*, 1985) localized at the *N*-terminus and carrying the binding site for C3b. These similarities between H and CR2 might suggest an even closer structural relationship than their common membership of a family of proteins containing several highly homologous subunits of about 60 amino-acids each (Weis *et al.*, 1986; Schulz *et al.*, 1986b). Although the epitopes of the four monoclonal antibodies therefore seem to be clustered in one relatively small part of the molecule, they differ with respect to their capacity to inhibit the binding of EAC3d to Raji lymphoblastoid cells (Table 2). The two antibodies reacting with the same or closely situated epitopes (1F8 and 2G7) did not inhibit rosette formation even in the presence of a second antibody (Table 2). The reason for this is not clear. Like the other epitopes, the ones recognized by 1F8 and 2G7 must be accessible on the surface of B-lymphoblastoid cells since these two antibodies stain unpermeabilized cells in immunofluorescence. With regard to their capacity to induce B-cell proliferation, there was no marked difference between these four monoclonal antibodies. As OKB7, a previously described monoclonal antibody to CR2, also binds to the 72,000 MW fragment of CR2 and stimulates B cells, whereas B2, another anti-CR2 monoclonal antibody, neither reacts with the 72,000 MW fragment nor induces B-cell proliferation (Myones & Ross, 1987; Nemerow *et al.*, 1985), it may be that antibodies to the C3d-binding 72,000 MW fragment of CR2 are good stimulants, whereas antibodies to other parts of CR2 are less active in this respect.

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