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Evidence for multiple sites of interaction in C3 for complement receptor type 2 (C3d/EBV receptor, CD21)

Multivalent but not monovalent CR2 ligands are required to elicit Raji cell proliferation as well as other B cell responses. It has been reported (C. Servis and J. D. Lambris, *J. Immunol.* 1989. 142: 2207) that the tetrameric peptide T-(C3^{1202–1214})₄, which represents the CR2-binding site in C3d, was able to support Raji cell growth. We show here that the tetrameric peptide T-(gp350^{19–30})₄, which contains the CR2-binding site in gp350 protein of EBV also induces Raji cell growth and this effect is inhibited by the monomeric peptides gp350^{19–30} and C3^{1201–1214}. We also investigated the nature of the interaction between C3 fragment and CR2 in order to explain the Raji cell growth-supporting effect exerted by C3. The following findings suggest that there are multiple sites in the C3 molecule able to interact with CR2: (1) both C3c and C3d immobilized on microspheres are able to bind to Raji cells through CR2. (2) soluble C3d inhibits to a greater extent the binding of CR2 to fixed C3d than to fixed C3b, which suggests the existence of additional CR2-binding sites within C3b not present in the C3d portion of the molecule; (3) synthetic peptides C3^{1187–1214}, C3^{741–757} and C3^{295–307} which represent regions of similarity in the C3 molecule bind specifically to CR2 on Raji cells and compete with each other for binding to the receptor and (4) preincubation of microtiter plate-fixed C3b with monoclonal or polyclonal anti-peptide antibodies (C3-9, anti-C3^{727–768}) recognize the N terminus of the α chain of C3 (including residues 741–757) inhibited CR2 binding. Therefore, these data suggest that the N terminus of the α chain of C3 is involved in binding to CR2.

1 Introduction

Human complement protein C3 plays a central role not only in complement activation but also in the regulation of the immune response. The physiological degradation products of C3 (C3b, iC3b, C3dg/C3d, C3a) are capable of exhibiting diverse biological functions as a result of their interactions with several cellular receptors [1–6]. One such receptor is the complement receptor type 2 (CR2), which is an integral membrane glycoprotein of 145 kDa [7–9] expressed on mature B cells, human pharyngeal epithelial cells, follicular dendritic cells, immature thymocytes and some T cell lines [3, 5, 6].

CR2 belongs to a family of complement regulatory proteins that contain a variable number of short consensus repeat units (SCR) and have the ability to bind to C3 and/or C4 fragments; CR2 consists of 15 or 16 SCR [10–12]. It is able to bind iC3b, C3dg and C3d fragments of C3 [1] while a low affinity binding has been reported for C3b [13]. A CR2-binding site on C3 has been localized to residues 1201–1214, within the C3d fragment [14].

CR2 also serves as the receptor for Epstein-Barr virus (EBV) [6, 15–17] and interferon- α [18]. The interaction of EBV with CR2 is mediated by the major envelope protein gp350 of EBV [19, 20]. The CR2-binding site on gp350 has been localized to the N terminus of the molecule (residues 19–31), a region which shows amino acid similarity with the CR2-binding site of C3d [21, 22]. The CR2-binding site on IFN- α was recently localized to residues 92–99 and was found to have sequence similarity to the CR2-binding site on C3d and gp350 [18].

CR2 has long been implicated in the regulation of B cell responses. The valency of the ligand interacting with CR2 appears to be important on the outcome of the response. Thus, polyvalent C3d/C3dg enhances proliferation of preactivated B cells and this effect can be inhibited by monovalent C3d/C3dg [23–25]. Polyvalent but not monovalent C3dg, is capable of priming human B lymphocytes for anti-IgM-induced proliferation and polyvalent CR2 ligands increase the anti-IgM-induced B cell intracytoplasmic Ca²⁺ influx, whereas monovalent ligands have an opposite effect [26, 27]. Similarly, multivalent synthetic peptides containing the CR2-binding site on C3d (tetrameric C3^{1202–1214}) support the growth of Raji cells under serum-free conditions and this effect can be inhibited by the

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Abbreviations: C3: Third component of complement iC3: C3 with hydrolyzed thioester bond C3b, iC3b, C3c, C3d and C3dg: Different degradation fragments of C3; CR2: Complement receptor type 2 gp350: Major envelope protein of EBV; CP: Control peptide; ms: Fluorescent microspheres

monovalent CR2-binding peptides C3¹²⁰¹⁻¹²¹⁴ and gp350¹⁹⁻³⁰ [22].

In this report, we show that, in addition to C3 peptides, multimeric peptides representing the CR2-binding site in gp350 protein of EBV (residues 19-30) also induce Raji cell growth, whereas the monomeric form of this peptide inhibits the Raji cell proliferative effect mediated by multivalent CR2 ligands. These results extend previous observations that multivalent CR2 ligands are required for Raji cell growth and confirm that the region in gp350 spanning residues 19-30 is involved in the binding to CR2.

The previously reported finding that C3 induces Raji cell growth [22, 28] was somewhat unexpected considering the fact that native C3 does not bind to CR2 and until now its fragments were thought to contain only one site of interaction for CR2. Therefore, we further investigated the nature of the interaction between C3 and CR2, in order to clarify the mechanism of C3-induced proliferation of Raji cells. Our results suggest that C3 gets converted to iC3 (by hydrolysis of the thioester bond) and/or degraded to its fragments (C3b and possibly iC3b) during culture, since C3b can both bind to CR2 and induce a proliferative response on Raji cells, as potent as C3. Furthermore, we demonstrate that the C3 molecule contains multiple sites of interaction with CR2, thereby explaining the growth-supporting effect of C3 on Raji cells.

2 Materials and methods

2.1 Reagents

Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp. (Freehold, NJ) porcine pancreatic elastase was obtained from Serva (Heidelberg, FRG), the coupling reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was purchased from Pierce (Rockford, IL) and fluorescent microspheres from Duke Scientific (Palo Alto, CA). All chemicals for peptide synthesis and analysis were purchased from Applied Biosystems (Warrington, GB).

2.2 Preparation of C3 fragments

C3 was isolated from EDTA plasma as previously described [29] except that the C3 was passed over Mono-Q HR10 as a final step of purification [30]. C3b was generated by limited trypsin digestion of C3, C3c and C3d by incubating C3 with 5% elastase (w/w) for 6 h at 37°C, as described elsewhere [30]. The purity of C3c and C3d was assessed by SDS-PAGE electrophoresis and by ELISA, using specific mAb anti-C3c (133H11) and anti-C3d (311). Aggregated C3 was prepared by glutaraldehyde treatment of C3, as described elsewhere [24].

2.3 Antibodies

Purified anti-CR2 mAb OKB7 was kindly provided by Dr. Patricia Rao (R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ). The hybridoma producing anti-

CR2 mAb HB-5 was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and the antibody was purified from ascites on a protein A column. The mAb NA1/34 (anti-CD1a) was purchased from Serotec (Bicester, GB) and the mAb 311 (anti-C3d) and C3-9 (anti-C3c) [31, 32] were kindly provided by Cytotech (La Jolla, CA) and Dr. E. Hack (University of Amsterdam, Amsterdam, The Netherlands), respectively. The anti-C3c mAb 133H11 was prepared according to standard procedures. Polyclonal antibodies against the synthetic peptides C3⁷²⁷⁻⁷⁶⁸ [30] and C3²⁹⁵⁻³⁰⁷ were raised in rabbits by s.c. injections of the peptides coupled to KLH [33] and purified from other serum proteins by affinity chromatography on a C3 or C3²⁹⁵⁻³⁰⁷-Sepharose column, respectively.

2.4 Cells

The cell line Raji, as CR2⁺ B cell line, was obtained from ATCC. Rael, a CR2⁻, EBV-carrying Burkitt lymphoma was generously donated by DR. E. Yefenof (Hadassa Medical School, Jerusalem, Israel). Tonsils were obtained from Bezirkspital (Rheinfelden, Switzerland). Tonsil cells were isolated from homogenized tissue washed five times with PBS and then lysed with lysing buffer (1% NP40 in PBS containing 5 mM EDTA, 2 mM PMSF, 1 μ M pepstatin, 1 μ M leupeptin and 2 mM diisopropyl fluorophosphate) for 1 h at 4°C at a concentration of 5×10^8 cells/ml. Insoluble material was removed by centrifugation and the SN were stored at -70°C prior to use.

2.5 Synthetic peptides

All peptides (Fig. 1) were synthesized by the standard solid-phase method of Merrifield [34] by using an Applied Biosystems (Foster City, CA) model 420A automated peptide synthesizer. Peptides coupled to the template KKKPGKEKG were prepared, as previously described [22]. After synthesis, the different peptides were released from the resin, extracted and purified by gel filtration on Sephadex G-25 and reversed-phase HPLC on a C₁₈ column. The purity of the peptides was assessed by HPLC and their amino acid composition as well as the peptide sequence were performed as previously reported [22]. The T₄-(4 α)_{II} peptide, a random sequence peptide synthesized on the same template, was kindly provided by Prof. M. Mutter, University of Lausanne. In order to prepare BSA-peptide conjugates, C3¹¹⁸⁷⁻¹²¹⁴ and a control peptide (CP) were synthesized with an additional cysteine in the N terminus and coupled to BSA through the free SH group of cysteine, using MBS as the coupling reagent [35]. Briefly, 4 mg BSA in 0.25 ml of 10 mM sodium phosphate buffer (PBS), pH 7.2, was reacted with 0.7 mg MBS (dissolved in dimethyl formamide). Free MBS was removed from the reaction product, BSA-MB, by spinning through a Bio-Spin P6 column (Bio-Rad Laboratories, Richmond, CA), equilibrated with PBS (pH 7.2). BSA-MB was then reacted with 5 mg of C3¹¹⁸⁷⁻¹²¹⁴ or CP peptide and the mixture was stirred for 3 h at room temperature. Peptide-BSA complexes were analyzed on 12% SDS-PAGE gels. Based on their electrophoretic mobilities, was calculated that approximately four to five molecules of C3¹¹⁸⁷⁻¹²¹⁴ and CP were coupled per molecule of albumin.

2.6 Radiolabeling

Iodination of proteins and peptides was accomplished by the iodogen and chloramine T-methods, respectively [36, 37]. Protein or peptide-associated ^{125}I were separated from free ^{125}I by gel filtration through a Bio-Spin P6 or PD10 (Pharmacia, Uppsala, Sweden) column, respectively.

2.7 Proliferation assay

Raji cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, penicillin (200 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and 10% heat-inactivated FCS at 37°C in a 5% CO_2 incubator. For the proliferation assays, Raji cells were thoroughly washed in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2 mM L-glutamine, penicillin (200 IU/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and 10 $\mu\text{g}/\text{ml}$ human transferrin (Sigma, St. Louis, MO), hereafter referred to as serum-free medium. Subsequently, Raji cells were resuspended in the serum-free medium and seeded in triplicate in 96-well microtiter plates at a final density of 1.5×10^3 cells/well (200 $\mu\text{l}/\text{well}$) in the presence or absence of different concentrations of C3, C3 fragments or synthetic peptides. After 5 days in culture, [^3H]dThd was added into each well and cell proliferation assessed by measuring the radioactivity incorporated into cellular DNA after 18 h of [^3H]dThd addition. The doubling time of cells, in cultures containing 10 $\mu\text{g}/\text{ml}$ C3, was found in earlier studies to be 36 h [28].

In order to investigate whether monomeric CR2-binding peptides are able to inhibit the Raji cell growth-supporting effect of multimeric CR2 ligands, serial dilutions of monomeric peptides were added together with suboptimal concentrations of C3, BSA-C3¹¹⁸⁷⁻¹²¹⁴, T-(C3¹²⁰²⁻¹²¹⁴)₄, T-(gp350¹⁹⁻³⁰)₄ peptides as stimulators of cellular growth and the experiments proceeded as described above.

2.8 Analysis of ligand binding to CR2 by three different assays

2.8.1 FCM analysis

C3, C3c, C3d, BSA, or synthetic peptides were coupled to fluorescent microspheres, as described elsewhere [38]. Briefly, equimolar amounts of C3, C3c, C3d or BSA (10 μM) or of synthetic peptides C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷, C3C3⁷⁴¹⁻⁷⁵⁷ or OVA¹⁷³⁻¹⁹⁶ (0.3 mM) were coupled to 50 μl of a 1% suspension of fluorescent microspheres by continuous stirring for 3 h at room temperature. Ligand-coated microspheres were subsequently incubated with 1% BSA in PBS to block free sites on microspheres. For CR2 ligand binding analysis, Raji cells (1×10^6) were incubated with 50 μl of a 0.1% suspension of protein- or peptide-coated microspheres at 4°C for 1 h in 100 μl of 3.5 mM veronal buffer containing 10 mM NaCl, 1% BSA, 3.5% dextrose, 0.2% sodium azide and 20 mM EDTA (BDVAE, pH 7.2, 6 mS at 22°C). Unbound microspheres were then separated from cell-associated microspheres by centrifugation through a cushion of 6% BSA-PBS at 200 \times g for 5 min. The pelleted cells were resuspended in PBS and examined for fluorescence with a FACScan flow cytometer (Becton Dickinson,

Mountain View, CA). The specificity of the binding was determined by preincubating the cells with 1 μg of anti-CR2 mAb OKB7, HB-5 or anti-CD1a mAb NA1/34 as a control, for 1 h at 4°C followed by incubation with saturating amounts of a polyclonal goat anti-mouse α serum (Southern Biotechnology Associates, Birmingham, AL) for 30 min at 4°C. Inhibition of peptide binding was also ascertained by preincubating the cells with 1 μg of aggregated C3 prior to addition of ligand-coated microspheres.

2.8.2 Inhibition of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ binding to Raji cells by CR2 binding peptides

Raji cells (1×10^6) were incubated for 30 min at 37°C with serial dilutions of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ as previously described [14]. Nonspecific binding was determined by preincubating replicate samples with a 100-fold excess of unlabeled ligand for 30 min before the addition of ^{125}I -peptide. Duplicate 50 μl samples were layered on 200 μl of Lenzol immersion oil (BDH, Poole, GB) and cell-associated ^{125}I -peptide was separated from free ^{125}I -peptide by centrifugation at 9000 \times g for 30 s in a Beckman (Palo Alto, CA) microcentrifuge. Inhibition of binding of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ to Raji cells by synthetic peptides was carried out by preincubating the cells with serial dilutions of unlabeled peptides at 37°C for 30 min prior to the addition of the radiolabeled ligand. The results were analyzed by the ligand nonlinear least squares curve-fitting program [39].

2.8.3 ELISA for binding of CR2 to C3 fragments

C3b or C3d (50 μl , 20 $\mu\text{g}/\text{ml}$) were fixed to microtiter wells in 0.1 M sodium hydrogen carbonate buffer (pH = 9.6), overnight at 4°C. After saturation with 1% BSA-PBS, serial dilutions of tonsil lysates (50 μl) were added and allowed to react for 1 h at 22°C. Bound CR2 was detected with ^{125}I -mAb HB-5. Inhibition of CR2 binding to microtiter-fixed C3b or C3d by fluid-phase C3c or C3d was carried out as follows. Fluid-phase C3c or C3d serially diluted (0.4–25 μM) were preincubated with 50 μl of tonsil lysates (1×10^7 cells/ml) for 1 h prior to the incubation with fixed C3b or C3d. In the case of inhibition of CR2 binding to fixed C3b by anti-C3 Ab, serially diluted anti-C3 Ab were preincubated with C3b fixed to microtiter plates before the addition of tonsil lysates. After the preincubation period, the assays were performed as described above.

3 Results

3.1 Raji cell growth is supported by multivalent but not monovalent CR2 ligands

Interaction of CR2 with its ligands induces B cell proliferative responses. As a model system we measured the growth of Raji cells cultured at low density under serum-free conditions, as previously described [22, 28]. CR2 ligands of monovalent or multivalent nature were used in order to assess their growth-supporting effect on Raji cells. These included C3, C3b, C3c, C3d, as well as peptides representing the CR2-binding sites in C3d or gp350 protein of EBV (Fig. 1). The different ligands were added to Raji cell cultures in the serum-free medium and DNA synthesis

PEPTIDE	SEQUENCE
C3 ¹²⁰¹⁻¹²¹⁴	PGKQLYNVEATSYA
C3 ¹¹⁹⁸⁻¹²¹⁴	WEDPGKQLYNVEATSYA
C3 ¹¹⁹¹⁻¹²¹⁴	TAKDKNRWEDPGKQLYNVEATSYA
C3 ¹¹⁸⁷⁻¹²¹⁴	KFLTAKDKNRWEDPGKQLYNVEATSYA
C3 ⁷⁴¹⁻⁷⁵⁷	SRSEFPESWLWNVEDLK
C3 ²⁹⁵⁻³⁰⁷	EDLVGKSLYVSAT
gp350 ¹⁹⁻³⁰	TGEDPGFFNVEI
OVA ¹⁷³⁻¹⁹⁶	VLVNAIVFKGLWEKAFKDEDTQAM
CP	CYLENGKETLQRADPPKTH
T-(C3 ¹²⁰²⁻¹²¹⁴) ₄	GKQLYNVEATSYA ← K GKQLYNVEATSYA ← K GKQLYNVEATSYA ← K GKQLYNVEATSYA ← K
T-(gp350 ¹⁹⁻³⁰) ₄	TGEDPGFFNVEI ← K TGEDPGFFNVEI ← K TGEDPGFFNVEI ← K TGEDPGFFNVEI ← K
T ₄ -(4α _{II})	LKKLANALATAAD ← K LKKLANALATAAD ← K LKKLANALATAAD ← K LKKLANALATAAD ← K

Figure 1. Amino acid sequence of the peptides used in this study. The numbering of amino acid residues of C3 peptides is based on the predicted sequence of C3 after subtracting the signal peptide sequence.

Table 1. Inhibition of the Raji cell growth-supporting effect of C3, T-(gp350¹⁹⁻³⁰)₄, T-(C3¹²⁰²⁻¹²¹⁴)₄ and BSA-C3¹¹⁸⁷⁻¹²¹⁴ by C3¹²⁰¹⁻¹²¹⁴ and gp350¹⁹⁻³⁰

Stimuli added	Inhibitor added (400 μM)			
	C3 ¹²⁰¹⁻¹²¹⁴	gp350 ¹⁹⁻³⁰	OVA ¹⁷³⁻¹⁹⁶	
	(cpm)			
C3	46742	3567	765	49631
BSA-C3 ¹¹⁸⁷⁻¹²¹⁴	31117	4167	257	33736
T-(gp350 ¹⁹⁻³⁰) ₄	25893	2816	202	31491
T(C3 ¹²⁰²⁻¹²¹⁴) ₄	27485	2790	514	29854

was measured (Fig. 2). T-(gp350¹⁹⁻³⁰)₄ and T-(C3¹²⁰²⁻¹²¹⁴)₄, two tetravalent peptides representing the CR2-binding site in gp350 and C3d, respectively, stimulate Raji cell growth in a dose-dependent manner. Although the two peptides have similar stimulatory capabilities at higher concentrations, T-(gp350¹⁹⁻³⁰)₄ is more efficient at lower concentrations. In contrast, the corresponding monomeric peptides, gp350¹⁹⁻³⁰ and C3¹²⁰¹⁻¹²¹⁴, as well as the unrelated multimeric peptide T₄-(4α_{II}) did not support Raji cell growth (Fig. 2A).

Another way to achieve multivalency was the coupling of peptides to BSA. BSA-C3¹¹⁸⁷⁻¹²¹⁴ supported Raji cell growth in a dose-dependent manner, as efficiently as C3, whereas 70–80 times higher concentration of monomeric C3¹¹⁸⁷⁻¹²¹⁴ was required to attain the same effect (Fig. 2B). The control peptide coupled to BSA (BSA-CP) did not induce Raji cell growth (Fig. 2B). The growth-supporting effect of monomeric C3¹¹⁸⁷⁻¹²¹⁴ was unexpected and is probably due to peptide aggregation as result of the hydrophobic nature of its N terminus (FLTT) [22]. This explanation is consistent with the results of the experiment shown in Fig. 2C where the monomeric overlapping peptides C3¹¹⁹¹⁻¹²¹⁴, C3¹¹⁹⁸⁻¹²¹⁴ and C3¹²⁰¹⁻¹²¹⁴ which lack the hydrophobic N terminus of C3¹¹⁸⁷⁻¹²¹⁴ failed to support Raji cell growth (Fig. 2C). Furthermore, monomeric C3¹²⁰¹⁻¹²¹⁴ and gp350¹⁹⁻³⁰ peptides inhibited the Raji cell growth-supporting effect of suboptimal doses of C3, T-(gp350¹⁹⁻³⁰)₄, T-(C3¹²⁰²⁻¹²¹⁴)₄ and BSA-C3¹¹⁸⁷⁻¹²¹⁴ (Table 1). Thus, monomeric peptides which are able to bind to CR2 can inhibit the Raji cell growth-supporting effect mediated by multivalent ligands.

3.2 C3 possesses multiple CR2-binding sites

The Raji cell growth-supporting effect of monomeric C3 is inconsistent with the finding that native C3 does not bind to CR2 and that it contains a single CR2-binding site. We assumed that C3 activation to iC3 or certain C3 fragments capable of binding to CR2 are generated during culture and that those activation products are responsible for supporting Raji cell growth. In order to address this issue, the Raji cell growth-supporting effect of C3 and its fragments C3b, C3c and C3d was assessed. The results show that C3 and C3b stimulated Raji cell growth in a dose-dependent manner (10–150 nM) and that C3c or C3d, at the same concentration range, displayed a slight or no effect, respectively (Fig. 2D). The cellular growth observed in the presence of high concentrations of C3c or C3d is probably due to protein aggregation. The fact that C3b stimulates Raji cell growth as efficiently as C3 supports our assumption that C3 activation products are involved in the C3-mediated cellular proliferation. Furthermore, those C3 activation products should contain both C3c and C3d fragments which, individually, cannot account for the high proliferative response. One plausible explanation could be that C3c and C3d each have one CR2-binding site and that the combination of both binding sites in C3 or C3b is responsible for CR2 cross-linking and subsequent cellular proliferation.

In order to assess the possibility that C3 contains multiple CR2-binding sites, competition ELISA experiments were performed. The results showed that fluid-phase C3d inhibits

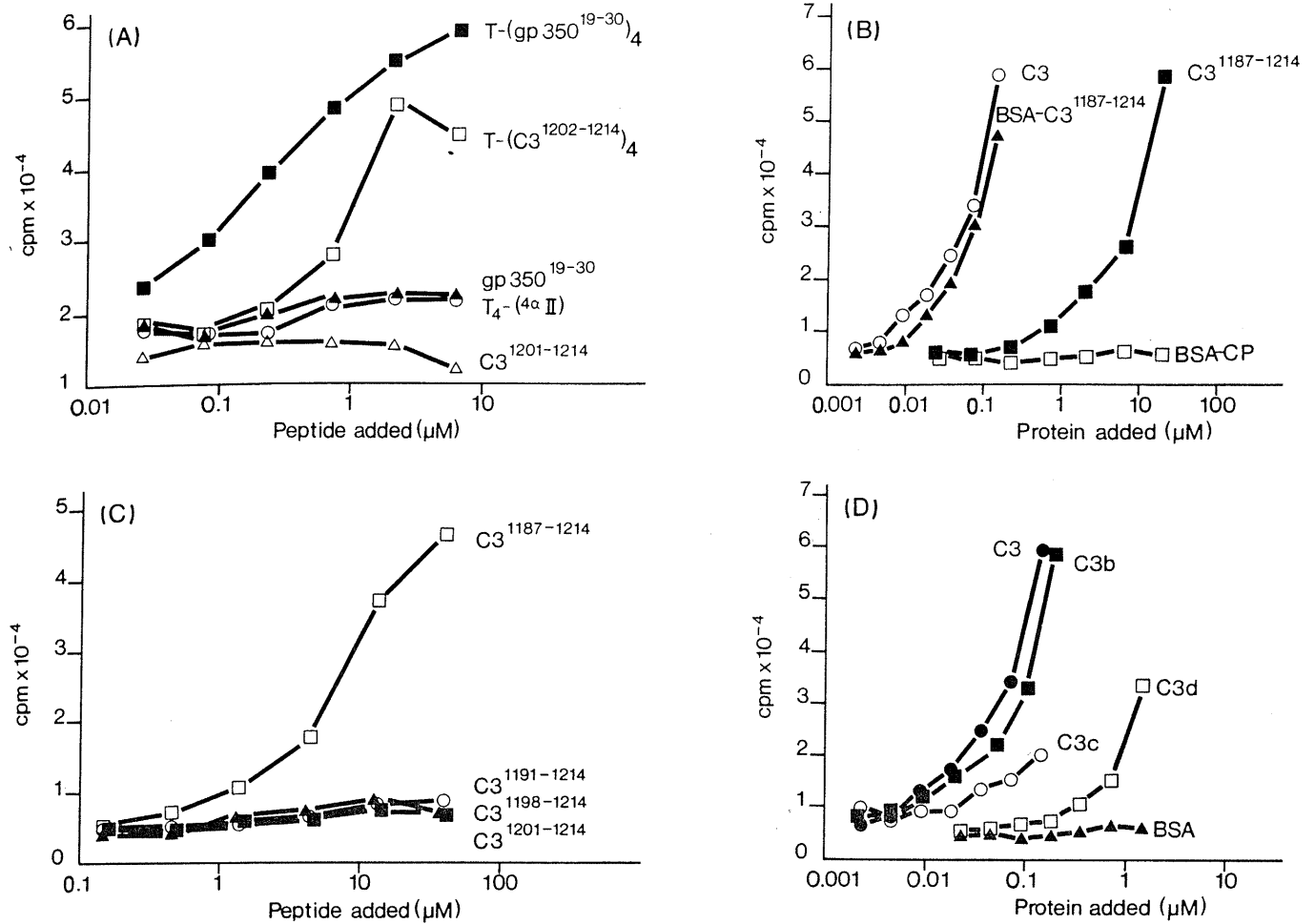


Figure 2. Raji cell growth-supporting effect by different CR2 ligands. Raji cells (1.5×10^3 cells/well) were cultured for 6 days in serum-free medium and different amounts of (A) T-(gp350¹⁹⁻³⁰)₄ (■), T-(C3¹²⁰²⁻¹²¹⁴)₄ (□), gp350¹⁹⁻³⁰ (▲), C3¹²⁰¹⁻¹²¹⁴ (△), or T₄-(4α II) (○); (B) C3 (○), BSA-C3¹¹⁸⁷⁻¹²¹⁴ (▲), C3¹¹⁸⁷⁻¹²¹⁴ (■), or BSA-Cp (□); (C) the overlapping CR2-binding peptides C3¹²⁰¹⁻¹²¹⁴ (■), C3¹¹⁹⁸⁻¹²¹⁴ (▲), C3¹¹⁹¹⁻¹²¹⁴ (○), or C3¹¹⁸⁷⁻¹²¹⁴ (□) and (D) C3 (●), C3b (■); C3c (○), C3d (□) or BSA (▲).

the binding of CR2 to fixed C3b and C3d in a dose-dependent manner (Fig. 3). Using ¹²⁵I-C3b and ¹²⁵I-C3d, it was found that the molar amount of C3d attached to the plates was four times greater than that of fixed C3b. The concentrations of fluid-phase C3d necessary to inhibit the binding of CR2 to microtiter plate fixed C3d and C3b by 50% (IC₅₀) were 2.3×10^{-6} M and 1.0×10^{-5} M, respectively (Fig. 3). This implies that almost 20 times more fluid phase C3d is required to inhibit to the same extent CR2-C3b as compared to CR2-C3d interactions. Therefore, the results from the ELISA experiments support the concept that C3 might possess additional CR2-binding sites outside the C3d fragment. Fluid-phase C3c did not significantly inhibit the binding of CR2 to either fixed C3b or C3d, which could be due to the low affinity of fluid-phase C3c for CR2.

Additional support of multiple binding sites in C3 for CR2 came from the analysis of the binding of C3 and its fragments C3c and C3d to CR2. C3, C3c and C3d were coupled to fluorescent microspheres and their binding to CR2 on Raji cells was quantitated by FCM. Although native C3 does not bind to CR2, when coupled to micros-

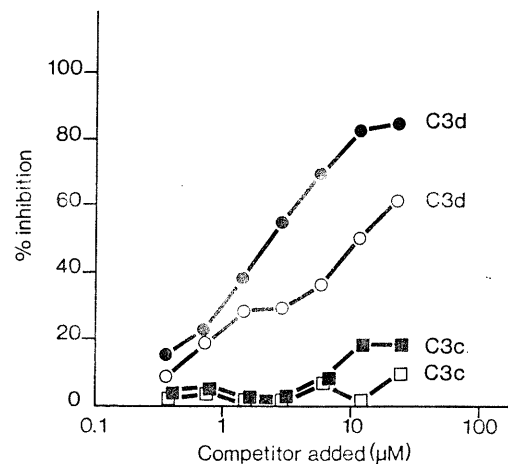


Figure 3. Inhibition of CR2 binding to microtiter plate-fixed C3b or C3d by fluid-phase C3c or C3d. C3b or C3d were fixed to microtiter plates and the binding of CR2 to fixed C3b (○, □) or C3d (●, ■) was carried out by preincubating tonsil lysates with serial dilutions (0.4–25 μM) of fluid-phase C3c (□, ■) or C3d (○, ●), as described in Sect. 2.8.2. Bound CR2 was detected with ¹²⁵I-mAb HB-5.

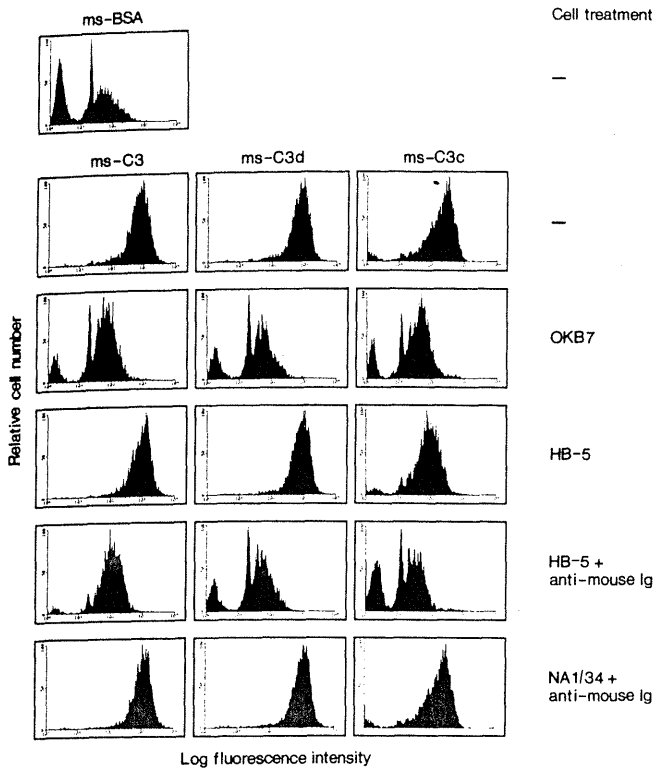


Figure 4. Binding of C3-, C3c- and C3d-coated microspheres to Raji cells. Fluorescent microspheres were coated with equimolar amounts of C3, C3c, C3d or BSA and their binding to Raji cells was determined by FCM. Specificity of the binding was assessed by preincubation of the cells with the anti-CR2 mAb OKB7 or HB-5 followed by a polyclonal anti-mouse α Ab. For comparison, mAb NA1/34 (anti-CD1a) was included as control.

pheres it expresses the CR2-binding site(s), hereafter C3 bound to microspheres will be referred to as ms-C3. Fluorescent microspheres (ms) bearing C3, C3c and C3d showed significant binding to Raji cells (>90%, 76% and >90% positive cells, respectively), whereas the binding of ms-BSA was minimal (Fig. 4). The specificity of ms-C3, ms-C3c or ms-C3d binding to CR2 was demonstrated by preincubating the cells with anti-CR2 mAb OKB7 and HB-5, in the presence or absence of a second polyclonal Ab anti-mouse α , prior to the addition of protein-bound microspheres. OKB7 totally abrogated ms-C3, ms-C3c and ms-C3d binding, whereas HB-5 inhibited 40% the binding of ms-C3c without reducing the binding of ms-C3 or ms-C3d. However, preincubation of Raji cells with HB-5 followed by the polyclonal Ab anti-murine α , resulted in complete abrogation of ms-C3, ms-C3c and ms-C3d binding to the cells (Fig. 4). Preincubation of Raji cells with an irrelevant, isotype-matched mAb control was without effect (Fig. 4). Additional proof that the binding to CR2 was specific was the finding that C3-, C3c- and C3d-coated microspheres did not bind to the CR2⁻ lymphoblastoid B cell line Rael (results not shown). Taken together the above data clearly indicate that there are at least two binding sites within the C3 molecule which are able to interact with CR2. One site is located within the C3d domain, as previously described [14], while the other(s) is (are) outside that domain, in the C3c fragment of the molecule.

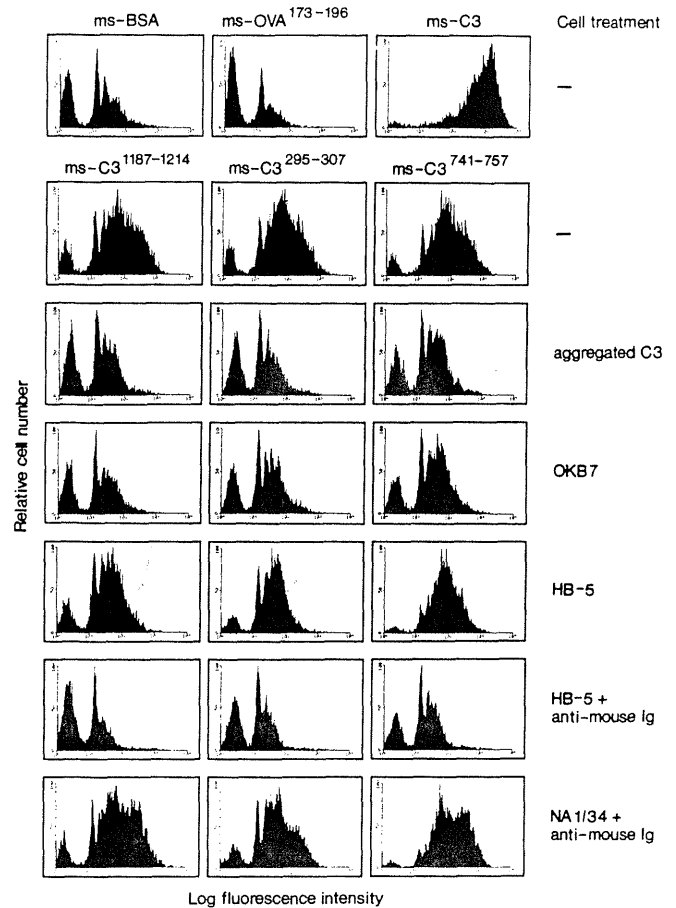


Figure 5. Binding of ms-C3¹¹⁸⁷⁻¹²¹⁴, ms-C3²⁹⁵⁻³⁰⁷ or ms-C3⁷⁴¹⁻⁷⁵⁷ to Raji cells. Fluorescent microspheres were coated with C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ or C3⁷⁴¹⁻⁷⁵⁷ peptides and their binding to Raji cells was analyzed by FCM. Specificity of the binding was assessed as described in the legend to Fig. 4.

3.3 Peptides from three regions of C3 bind to CR2

Computer analysis of the C3 sequence revealed two regions in C3 within amino acid sequences similar to the CR2-binding site in C3d; these are the regions spanning residues 295-306 (β chain) and residues 744-755 (α chain) (Table 2 and Fig. 8). In order to assess the involvement of these C3 segments in the binding to CR2, the peptides C3¹¹⁸⁷⁻¹²¹⁴, C3⁷⁴¹⁻⁷⁵⁷ and C3²⁹⁵⁻³⁰⁷ (Fig. 1), which cover the segments of similarity described above, were synthesized, coupled to fluorescent microspheres and their binding to Raji cells assessed by FCM. C3¹¹⁸⁷⁻¹²¹⁴, C3⁷⁴¹⁻⁷⁵⁷ and C3²⁹⁵⁻³⁰⁷ coupled to microspheres showed significant binding to Raji cells (40%-45% positive cells) (Fig. 5). The binding of fluorescent microspheres coated with BSA or the unrelated peptide OVA¹⁷³⁻¹⁹⁶ was insignificant, whereas the binding of microspheres coated with C3 was higher than that observed with the synthetic peptides (90% positive cells; Fig. 6). The binding of C3¹¹⁸⁷⁻¹²¹⁴, C3⁷⁴¹⁻⁷⁵⁷ and C3²⁹⁵⁻³⁰⁷ coupled to microspheres could be inhibited totally by aggregated C3 and by nati-CR2 mAb OKB7. A partial inhibition was observed with anti-CR2 mAb HB-5 alone, whereas total inhibition occurred when the latter antibody was used in conjunction with polyclonal Ab anti-murine α (Fig. 5). Preincubation of the cells with mAb NA1/34 in the presence of polyclonal Ab anti-murine α was without any effect (Fig. 5). These results indicate that C3¹¹⁸⁷⁻¹²¹⁴,

Table 2. Amino acid sequence similarity between the identified CR2-binding site in C3d with other segments of C3, the gp350/220 protein of EBV and IFN- α

	Residues	Sequence ^{a)}
CR2-binding site in C3d	1199-12101	ED . P G K Q L Y N V E A
β Chain of human C3	295- 306	** L V ** S ** . * S *
α Chain of human C3	744- 755	* F . * E S W * W * * * D
CR2-binding site in gp350	21- 30	** * * . F F * * * I
CR2-binding site in IFN-	87- 98	F T . E L Q * * N D L * *

a) Identical residues are denoted by the symbol * while the gaps, introduced for maximal sequence alignment, are denoted by a period.

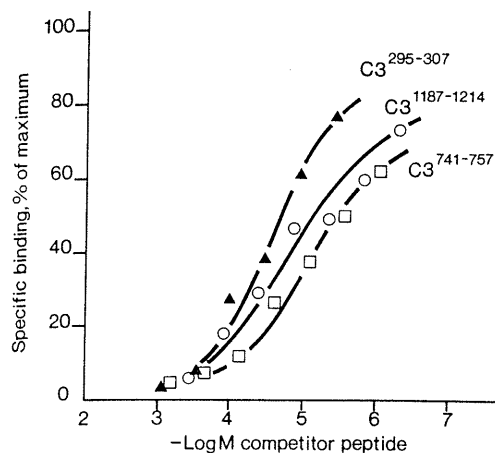


Figure 6. Inhibition of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ binding to Raji cells by unlabeled C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ or C3⁷⁴¹⁻⁷⁵⁷ peptides. Raji cells were preincubated with various concentrations of C3¹¹⁸⁷⁻¹²¹⁴ (○), C3²⁹⁵⁻³⁰⁷ (▲) or C3⁷⁴¹⁻⁷⁵⁷ (□) at 37°C for 30 min, before the addition of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ (0.1 nmol) and the binding assay was performed as described in Sect. 2.8.2. The specific uptake represents 74% of the total ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ binding and the standard deviations range between 1%–7% of the specific binding values. These results are representative of one experiment out of six performed.

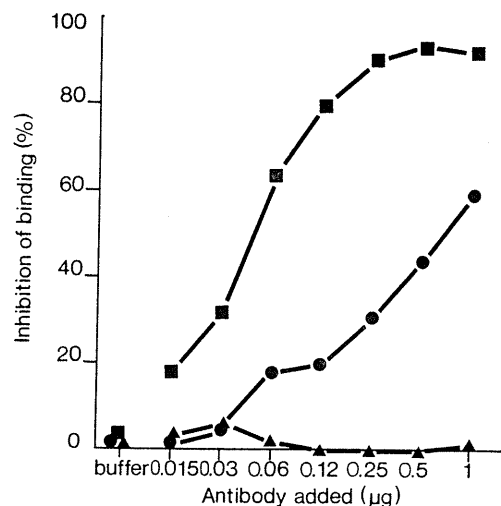


Figure 7. Inhibition of CR2 binding to microtiter plate-fixed C3b by anti-C3 antibodies. Binding of CR2 to C3b fixed on ELISA plates was carried out by preincubating C3b with serial amounts (0.015–1 μg) of mAb C3-9 (■), mAb 133H11 (▲) or the polyclonal anti-C3⁷²⁷⁻⁷⁶⁸ peptide Ab (●). Bound CR2 was detected with ^{125}I -mAb HB-5.

C3⁷⁴¹⁻⁷⁵⁷ and C3²⁹⁵⁻³⁰⁷ peptides bind specifically to CR2 present on Raji cells.

Whether C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ and C3⁷⁴¹⁻⁷⁵⁷ peptides compete with each other for CR2 binding was assessed by measuring the percentage of labeled peptide bound as a

function of the concentration of unlabeled peptide(s) in a series of competitive displacement experiments. Unlabeled C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ and C3⁷⁴¹⁻⁷⁵⁷ compete for the binding of ^{125}I -C3¹¹⁸⁷⁻¹²¹⁴ to Raji cells. The K_d values are 0.55×10^{-5} M, 1.8×10^{-5} M and 2.6×10^{-6} M for C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ and C3⁷⁴¹⁻⁷⁵⁷, respectively (Fig. 6). Similar results were obtained when the peptide C3²⁹⁵⁻³⁰⁷ was used as the labeled ligand (data not shown).

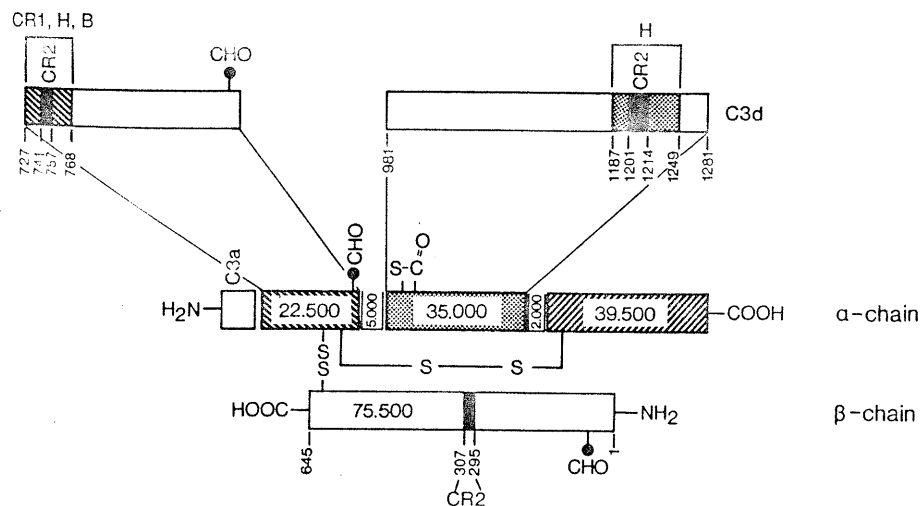


Figure 8. A schematic representation of the C3 molecule showing the position of CR2-binding peptides and the regions of C3 which has been shown to bind CR1, factor H and factor B.

Thus, the data of these peptide-binding assays show that C3⁷⁴¹⁻⁷⁵⁷ peptide binds to the cells with an affinity value approximately 2 or 7 times higher than that expressed by C3¹¹⁸⁷⁻¹²¹⁴ or C3²⁹⁵⁻³⁰⁷, respectively, which suggests that the region of C3 which includes amino acid residues 7141-757 might play an important role in the binding of C3 to CR2.

3.4 Evidence that the region of C3 spanning residues 741-757 is involved in CR2 binding

In order to further investigate if the C3 regions spanning residues 295-307 and 741-757, when in the native conformation of C3 fragments, are involved in CR2 binding, anti-peptide antibodies to these regions were generated. Although the generated antibodies recognized the peptide that was used as immunogen only the anti-C3⁷²⁷⁻⁷⁶⁸ antibody recognized native C3 fragments. The specificity of the anti-C3⁷²⁷⁻⁷⁶⁸ antibody was defined by using C3 fragments and it was found that it reacts with fluid-phase C3b, iC3b and C3c but not with C3d of C3 (Becherer et al., submitted for publication). Affinity-purified anti-C3⁷²⁷⁻⁷⁶⁸ antibody was able to significantly inhibit the binding of CR2 to fixed C3b (Fig. 7). The effect of this antipeptide antibody was compared to that of mAb C3-9 which is antibody that also recognizes a region in the N terminus of the α -chain of C3 [30, 40].

Preincubation of fixed C3b with either antibody inhibited CR2 binding in a dose-dependent manner (Fig. 7). In contrast, the anti-C3c mAb 133H11 did not show any inhibitory effect (Fig. 7). Taken together, these data indicate that the region of C3 which includes amino acid residues 741-757, is involved in CR2 binding. Similar experiments to ascertain whether or not the region of C3 spanning residues 295-307 mediates CR2 binding could not be performed because the polyclonal anti-C3²⁹⁵⁻³⁰⁷ antibody raised, while reacting strongly with C3²⁹⁵⁻³⁰⁷, it showed no reactivity with C3 and its fragments.

4 Discussion

Functional studies have shown that interaction of CR2 with its ligands results in modulation of the growth of B lymphocytes and lymphoblastoid B cell lines. In agreement with previous studies [22-25, 41], we also demonstrate that the valency of the CR2-ligand plays an important role in the outcome of the response. Thus, the results herein show that multivalent T-(gp350¹⁹⁻³⁰)₄, T-(C3¹²⁰²⁻¹²¹⁴)₄ and BSA-C3¹¹⁸⁷⁻¹²¹⁴ peptides support Raji cell proliferation and that the monomeric gp350¹⁹⁻³⁰ and C3¹²⁰¹⁻¹²¹⁴ peptides not only do not induce Raji cell growth, but also inhibit the growth-supporting effect of the multimeric CR2-ligands.

Evidence for two forms of human CR2 has been found in a variety of transformed human B cells, including Raji cells [42]. Similar to the *neu* oncogene protein [43], these two forms of human CR2 might be randomly distributed or clustered within the cell membrane. The existence of these two different CR2 arrangements might explain some of the results presented here, *i.e.* template-coupled peptides might only cross-link clusters of CR2, whereas when conjugated to BSA, the CR2-binding peptides are more

distant and could, therefore, also cross-link non-aggregated forms of CR2, which may lead to an increase in cellular growth.

Our results also support previous observations that the synthetic peptide gp350¹⁹⁻³⁰ is involved in CR2 binding since a multimeric form of this peptide, T-(gp350¹⁹⁻³⁰)₄, induces Raji cell growth whereas the monomeric peptide, gp350¹⁹⁻³⁰, inhibits the Raji cell growth-supporting effect of C3 and polyvalent CR2-ligands including T-(gp350¹⁹⁻³⁰)₄, BSA-C3¹¹⁸⁷⁻¹²¹⁴ and T-(C3¹²⁰²⁻¹²¹⁴)₄. In addition, our data indicate that C3d and gp350 bind to the same domain in CR2, since peptides spanning the corresponding CR2-binding sites could block each other's ability to induce cellular growth. This indication is supported by the recent finding that both C3dg and gp350 bind to the same region in CR2 (SCR1 and 2) [44].

The stimulatory effect of the synthetic peptide C3¹¹⁸⁷⁻¹²¹⁴ cannot be explained on the basis of its monovalent nature. However, the fact that the overlapping peptides C3¹²⁰¹⁻¹²¹⁴, C3¹¹⁹⁸⁻¹²¹⁴ and C3¹¹⁹¹⁻¹²¹⁴, which lack the hydrophobic N terminus of C3¹¹⁸⁷⁻¹²¹⁴, did not induce Raji cell growth, supports the explanation provided in an earlier report [22] that C3¹¹⁸⁷⁻¹²¹⁴ aggregation through its hydrophobic N terminus (FLTT), might occur.

In order to explain the mitogenic effect of monomeric C3 on the growth of Raji cells, it is first necessary to establish that C3 binds to CR2. The fact that C3b supports Raji cell growth to the same extent as C3 supports the possibility that native C3 has been either converted to iC3 or cleaved to C3b and/or iC3b by cell derived proteases [45, 46]. In fact, in experiments in which we analyzed the presence of the neoantigenic epitope, recognized by mAb C3-9 and 130 (both antibodies inhibit the binding of iC3 to CR2), in C3 after 6 days of culture we found that a significant amount of C3 expresses these epitope (Lao and Lambris, unpublished observations). Complete cleavage to C3c and C3d fragments is excluded, due to the lower stimulation observed with these fragments. An observation of interest was the finding that fluid-phase C3d inhibited to a greater extent the interaction of CR2 to immobilized C3d as compared to immobilized C3b. This result suggests that there might be CR2-binding sites in C3b/iC3b which are not present in the C3d portion of the molecule. Further evidence that, indeed, this is true was provided by experiments which showed that fluorescent beads coated with C3c (ms-C3c) are able to bind specifically to CR2 on Raji cells.

Our results indicate that HB-5, in contrast to OKB7, binds to a region of CR2 quite apart from that involved in C3, C3c and C3d binding, since HB-5 inhibits only partially the binding of ms-C3c to Raji cells, whereas its effect on ms-C3 and ms-C3d binding is negligible. This indication is supported by the recent finding that the epitope recognized by HB-5 is localized to a region including SCR-3, SCR-4 and the first third of SCR-5, whereas OKB7 as well as C3d and gp350 of EBV bind to SCR-1 and SCR-2 of CR2 [44, 47]. Previous studies failed to find C3c binding to CR2 [48]. Experimental differences may account for these discrepancies. The binding assays reported here were performed under optimal conditions, (*i.e.* low ionic strength buffer) and the binding was detected by FCM analysis rather than by rosetting. On the other hand, the inhibition of CR2

binding to ELISA-fixed C3b or C3d by fluid-phase C3c was negligible, which suggests that fluid-phase C3c has low affinity for CR2 and that C3c binding to CR2 might only be detected when the ligand is coupled to microspheres and therefore interacting with CR2 in a multivalent manner through a newly exposed site in the surface-bound molecule. The finding that a polyclonal anti-C3c antibody inhibited EC3bi rosette formation with Raji cells [49] supports our results that there are CR2-binding sites within C3c.

The observation that there are two regions in C3 which show amino acid sequence similarity with the CR2-binding site on C3d provided further evidence that C3 might interact with CR2 via multiple sites. When the peptides containing the three similar regions were synthesized and coupled to fluorescent beads, they were able to bind to CR2 on Raji cells in a specific manner, since the binding was inhibited by aggregated C3, mAb OKB7 or mAb HB-5 in combination with the polyclonal Ab anti-murine α . Further investigation of the peptide(s) interaction with Raji cells revealed that the three peptides C3¹¹⁸⁷⁻¹²¹⁴, C3²⁹⁵⁻³⁰⁷ and C3⁷⁴¹⁻⁷⁵⁷ compete with each other for the binding to the cells which indicates that they bind to the same or similar sites in CR2. C3⁷⁴¹⁻⁷⁵⁷ binds with the highest affinity, whereas C3²⁹⁵⁻³⁰⁷ binds with the lowest affinity to Raji cells. C3¹¹⁸⁷⁻¹²¹⁴ revealed a $K_d = 0.55 \times 10^{-5}$ M, a value similar to that reported for monomeric C3dg [50]. The finding that C3⁷⁴¹⁻⁷⁵⁷ binds with higher affinity than C3¹¹⁸⁷⁻¹²¹⁴ to CR2, whereas monomeric C3c does not inhibit CR2 binding to C3 fragments, indicates that the region of C3c spanning residues 741-757 is masked in the native C3c molecule. However, this region might be exposed in iC3, C3b, iC3b or when C3c is fixed to a solid support, such as microspheres, thereby explaining the observed results.

The above data clearly show that C3 contains three regions capable of binding to CR2 (see Fig. 8 for a schematic representation of these sites in C3). Whether or not they all contribute to receptor interaction in the intact molecule needs further elucidation. However, the binding that mAb C3-9 or polyclonal Ab anti-C3⁷²⁷⁻⁷⁶⁸, which recognize the N terminus of the α chain of C3 [30, 32] including residues 741-757 inhibit the binding of CR2 to ELISA-fixed C3b, indicates that this region is involved in the binding of C3 to CR2. Within this domain (N terminus of the α chain), two additional binding sites for CR1 and factor H have also been localized [30, 32]. Our experiments, however, do not exclude the possibility that all three regions described above constitute areas of larger binding site; it is found in other systems that the surfaces of individual proteins forming recognition sites are between 6-10 nm² [51].

The "multiple site" model for the interaction of C3 fragments with CR2 is similar to that demonstrated for C3b binding to factors H and B [3, 52-54], two other members of the family of complement regulatory proteins. This model can also be extended to the interaction of gp350/220 with CR2. The findings that mouse CR2, despite binding to human C3d, cannot bind EBV together with the data showing that the binding site in gp350 of EBV for human CR2 has sequence similarity to the CR2-binding site in C3d, suggests that CR2 possess additional interaction sites for EBV. In support of this are (a) the Scatchard analysis of gp350 binding to CR2, showing both high- and low-affinity

receptor binding sites [55] and (b) the inhibition of EBV but not of C3d binding to CR2 and *vice versa* by several anti-CR2 antibodies [56]; this is in contrast to OKB7 antibody that inhibits the binding of both ligand. Since the human CR2-binding site in mouse C3 is conserved [3], one could speculate that for efficient binding of EBV to CR2 both sites are necessary. Recent data by Molina et al. using chimeric CR2 molecules constructed by exchanging mouse SCR1-4 as well as synthetic peptides confirmed that CR2 has two different EBV-binding sites [57]. In addition the same authors and Martin et al. [58], using chimeric hu/mo CR2 molecules were able to dissect the EBV-, C3d- and OKB7-binding sites and to show that these sites differ, although they may share some structural elements.

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