Characterization of C3dg Binding to a Recess Formed Between Short Consensus Repeats 1 and 2 of Complement Receptor Type 2 (CR2; CD21)

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*J Immunol* 1998; 161:4604-4610; ;
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Characterization of C3dg Binding to a Recess Formed Between Short Consensus Repeats 1 and 2 of Complement Receptor Type 2 (CR2; CD21)\(^1\)

Wolfgang M. Prodinger, Michael G. Schwendinger, Jürgen Schoch, Maria Köchle, Clara Larcher, and Manfred P. Dierich

To allow for a better characterization of the ligand binding structures of human complement receptor type 2 (CR2; CD21), we have established an IgG1 \(\kappa\) mouse mAb, FE8, that interferes efficiently with binding of C3dg and EBV to CR2. In contrast to mAb OKB7, the only well-characterized mAb with similar specificity, mAb FE8 blocked binding of soluble C3dg or particles carrying multiple copies of surface-bound C3dg to CR2 or induced complete removal of these ligands from the receptor. In vitro EBV infection of B lymphocytes, on the other hand, was abrogated by mAbs FE8 and OKB7 with similar dose-response characteristics. As FE8 was shown to recognize a discontinuous epitope, a series of overlapping peptides derived from SCR1 and -2 and immobilized on cellulose was screened with FE8. The results suggest that up to five discontinuous sequences contributed to the epitope. The sequence 63-ENFKYS-69, located between the two SCR units, reacted most intensively. Two other sequences, 16-YYSTPI-21 and 105-NGNKSVWCQANN-116, are located between Cys\(^1\) and Cys\(^2\) of SCR1 and around Cys\(^3\) of SCR2, respectively. Based on the solution structure for two factor H SCRs, a three-dimensional model of SCR1 and -2 was generated. The FE8 binding peptide sequences were located in relative proximity to each other, bounding the recess formed between SCR1 and -2. This potential of mAb FE8 is currently unique and may be exploited for interfering with conditions of unwanted recognition of C3dg-coated structures by the immune system. *The Journal of Immunology*, 1998, 161: 4604–4610.

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### Notes

1. **Human CR2** (CD21) is a membrane glycoprotein of 145 kDa predominantly expressed on mature B lymphocytes (1) and follicular dendritic cells (2) and to a much lesser extent on peripheral T lymphocytes (3), thymocytes (4), or astrocytes (5). The function of CR2 has been most extensively studied for B lymphocytes (for a review, see Ref. 6). CR2 is the receptor for C3dg and (with lower affinity) for iC3b, fragments of C3 that are generated during complement activation and covalently deposited on surfaces, e.g., of pathogens. On B cells, CR2 forms a noncovalent receptor complex with the B cell-specific CD19 molecule and CD81, which is broadly expressed among hemopoietic cells (7). Coligation of this complex and the BCR complex lowers the threshold for B cell activation substantially (8). The extent of this effect is exponentially correlated to the number of C3dg residues on the specific Ag (9).

Of pathophysiologic relevance, EBV infection of human cells requires attachment to CR2 as a first step. The binding is mediated via the envelope glycoprotein gp350/220, which shares sequence homology with C3dg (10). The gp350/220 interacts with the same N-terminal pair of short consensus repeats (SCRs) as does C3dg (11). Furthermore, the low affinity Fc\(\epsilon\) receptor CD23 has been established as a ligand of CR2 with a glycosylation-dependent binding site on SCR8, with SCR1 and -2 additionally contributing to CD23 binding (12). The interaction of CD23 and CR2 is considered important for the production of IgE (13) and for survival of germinal center B cells (14).

CR2 is a member of the family of C3b and/or C4b binding proteins (15), and its extracellular part is formed by 15 or, as a result of alternative splicing, 16 SCRs. These structural units of about 60 amino acids share a frame of several strictly conserved amino acids, most importantly four cysteine residues that are linked by disulfide bonds in a Cys\(^1\)-Cys\(^2\) and Cys\(^3\)-Cys\(^4\) manner. The solution structure for individual SCRs (16) or a pair of SCRs (17) from factor H has been determined by \(^1\)H nuclear magnetic resonance. Based on these findings, a \(\beta\)-sandwich structure of two separate \(\beta\)-strands on one face and three hydrogen-bonded \(\beta\)-strands on the other has been suggested for single SCR units (18).

The structure of the two N-terminal SCRs involved in ligand binding has extensively made use of the mouse mAb OKB7, which was shown early to inhibit CR2-dependent EAC3d rosetting of Raji cells (19) and to block infection of B cells with EBV (20). OKB7 has also been employed to demonstrate an epitope dependence regarding effects of CR2 mAbs on B cell proliferation. However, this feature of OKB7 vs other nonblocking mAbs as HB5 (1) is not consistently defined. On the one hand, OKB7, but not HB5, induced proliferation of B cells from peripheral blood in the presence of T cells (21) or of resting tonsil B cells together with PMA (22). When cross-linked together with anti-surface IgM, OKB7, but not HB5, had an enhancing effect on the c-fos transcription in resting tonsil B cells (23). OKB7, but not HB5, was reported to induce mobilization of intracellular Ca\(^{2+}\) without requirement for cross-linking in peripheral blood B cells (24). Similarly, OKB7, but not HB5, induced Ca\(^{2+}\) mobilization when added to CR2+ HBP-ALL T lymphoblastoid cells or to purified peripheral blood T
cells, although the latter effect appears to be limited (25). In contrast, others have underlined that CR2 signaling relies on coligation with the BCR and could be obtained with polymeric C3dg as well as with the nonblocking mAb HB5 (8, 26). Consistent with this view, cross-linking CR2 with the BCR via CD23 lowered the amount of anti-IgM necessary for B cell proliferation in a similar manner by 2 orders of magnitude (27). Thus, the nature of this reported epitope dependence remains unexplained and in part contradictory.

Since Lowell and coworkers (11) showed that the two N-termini of CR2 are both essential and together are sufficient for binding of C3dg, EBV, and OKB7, the binding sites for the three ligands have been dissected in more detail. By inhibition of EAI/C3b rosette formation with peptides, Molina et al. (28) suggested that the SCR1 sequence 10-LNGRIS-15 and the SCR2 sequence 84-GST PYRGDSVTFA-97 importantly contribute to C3dg binding, with a potential further contribution of peptide 99-KTNFS MNGNKSVW-111. Furthermore, the rat mAbs 7G6 and 4E3, which inhibit binding of mouse or human C3dg to mouse CR2 independently (29, 30), have their epitopes in corresponding parts of mouse CR2 (28, 31).

As none of the seven inter-Cys sequences of human CR2 in SCR1 and -2 is indispensable for EBV binding when replaced with the corresponding sequence from mouse CR2, Martin et al. suggested that the overall conformation of SCR1 and -2 is primarily responsible for gp350 binding (31). However, by introduction of two discontinuous amino acid substitutions, mouse CR2 could be made a receptor for EBV, but still does not bind OKB7 (32).

A further characterization of the CR2 ligand binding site has been hampered in part by the restricted repertoire of blocking mAbs to human CR2. Additionally, although mAb OKB7 inhibited EAC3d rosetting with CR2-expressing cells to >95% in our hands, it was rather inefficient in blocking CR2-dependent complement activation on Raji cells (33). This together with its current unavailability due to unknown reasons prompted us to attempt the generation of more effective mAbs that might allow another view on the structural determinants of ligand binding to CR2. To this end we employed a baculovirus-derived soluble CR2 protein truncated after SCR4 (34) as an immunogen and inhibition of the binding of FITC-labeled, C3d-coated agarose microbeads to CR2 as a screening method, which proved to be a successful strategy.

Materials and Methods

Abs, cell lines, and viruses

The following (purified) mAbs were used: HB5, anti-CR2 (1) (American Type Culture Collection (ATCC), Manassas, VA); OKB7, anti-CR2 (19) (Ortho, Raritan, NJ); MCA-664, anti-CR2 (clone B-E5; Serotec, Oxford, U.K.); 72A1, anti-gp350/220 of EBV (35) (ATCC); TIB191 (IgG1), anti-EBNA (Ortho, Raritan, NJ); INN-81 (IgG2b), anti-FSH (36); and VD3 (IgG1/2a) and biotinylated IIC5 (IgG1), both anti-factor H (37) and used as isotype controls.

The polyclonal Abs used were anti-C3d, anti-C3d-FITC, and anti-mouse-PE from rabbit serum; swine anti-rabbit-FITC, and goat anti-mouse-PE (all from Dako, Glostrup, Denmark).

The B lymphoblastoid Raji cell line (EBNA, CR2+, surface IgM) was obtained from ATCC and maintained in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) with 10% FCS (Biologic Industries, Kibbutz Beth Haemek, Israel), 2 mM l-glutamine, and penicillin/streptomycin at cell densities between 2 and 10 × 10^6/ml. Transforming EBV of the B9-5 strain (38) was obtained as cell culture supernatant as previously described (39).

Preparation of a truncated soluble CR2 molecule for immunization

A recombinant baculovirus encoding the signal peptide, the first four SCRs of CR2, and a C-terminal His tag was used for production of recombinant soluble CR2 (rsCR2.1–4) as described previously (34). The rsCR2.1–4 protein used for immunization was >90% pure as judged by SDS-PAGE and silver staining.

Generation and screening of hybridoma clones

Female 8-wk-old BALB/c mice were immunized s.c. with 8 μg of purified rsCR2.1–4 Ag emulsified in CFA. The mice were boosted with 8 μg of rsCR2.1–4 in IFA s.c. after 4 wk and with 75 μg of rsCR2.1–4 i.p. after an additional 4 wk, i.e., 3 days before being sacrificed. Spleen cells were fused with the P3X63Ag8.653 (40) mouse myeloma cells in a ratio of 1:1 (spleen lymphoblasts to myeloma cells) according to the procedure of Fazekas et al. (41). Hybridoma supernatants reactive either in ELISA with rsCR2.1–4 (34) and/or in FACS analysis with Raji cells were further tested for inhibition of binding of C3d-coated microbeads as described below. Clones of interest were subcloned twice by limiting dilution technique. IgG purified from ascitic fluid by affinity chromatography on protein G-Sepharose (Pharmacia, Uppsala, Sweden) was eventually coupled to N-hydroxy-succinimido-biotin (Sigma, St. Louis, MO) by standard methods.

Binding of C3d-coated agarose microbeads to CR2

FITC-labeled agarose microbeads of 1-μm diameter were coated with C3d by incubation with normal human serum followed by trypan blue as previously described (42). Analysis by SDS-PAGE (under nonreducing conditions) of the proteins covalently bound to the microbeads showed that >90% consisted of a 32-kDa Mr molecule that stained with anti-C3d Ab and could be eluted with 1 M hydroxyamine as previously described (43).

To test the C3d-binding potential of mAbs, Raji cells washed in FACS buffer (PBS, 1% BSA, and 0.1% sodium azide), devoid of sodium azide, were incubated with mAbs for 15 min at room temperature at a density of 5 × 10^6 cells/50 μl. Without washing the cells, C3d microbeads resuspended in 10 μl of PBS were added at a concentration previously determined to produce half-maximal binding to unblocked cells. After an additional incubation period of 30 min at room temperature, 200 μl of FACS buffer was added, and the samples were analyzed immediately by FACS. Cells bearing beads attached were distinguished by their bright green fluorescence. After acquiring 10,000 cells, binding was quantified as the percentage of cells carrying beads, which typically ranged between 20 and 40% for unblocked Raji cells. Unspecific binding was assessed by the use of FITC-labeled agarose microbeads lacking C3d and was consistently <4%. Specific binding of C3d microbeads in the presence of mAbs was expressed as the percentage of maximal specific binding observed without mAb.

Competition of mAbs for binding of soluble C3dg

C3dg was purified from pooled human plasma as described by Vik and Fearon (44). Raji cells (1 × 10^7) were washed with FACS buffer and preincubated with 20 μg/ml C3dg in PBS and 1% BSA for 20 min at 25°C before mAbs in increasing concentrations were added for a further incubation of 20 min at either 4 or 25°C. After washing twice with 1 ml of FACS buffer bound C3d was detected with FITC-labeled rabbit anti-C3d, and bound anti-CR2 mAbs were stained with PE-conjugated goat anti-mouse Ig. Immunofluorescent signals were quantified by flow cytometry using a FACScan (Becton Dickinson, Hialeah, FL).

Competition between OKB7 and FE8 for binding to CR2 was assessed by preincubating Raji cells washed in FACS buffer with 10 μg/ml of unlabeled mAb for 15 min on ice. Biotinylated FE8 was added at a concentration that had been determined to produce 30% maximal staining for an additional 15 min. After incubation with cychrome 5 PE-labeled streptavidin (Sigma), immunofluorescence was measured by flow cytometry.

Inhibition of in vitro infection of B lymphocytes with EBV

The ability of anti-CR2 mAbs to inhibit EBV-induced cell transformation of B cells was tested for peripheral blood B lymphocytes and isolated tonsil B cells. PBL were isolated from 20 ml of heparinized blood from EBV-positive normal human donors by Ficoll-Hypaque gradient centrifugation and bound anti-CR2 mAbs were stained with PE-conjugated goat anti-mouse Ig. Immunofluorescent signals were quantified by flow cytometry using a FACScan (Becton Dickinson, Hialeah, FL).

Competition between OKB7 and FE8 for binding to CR2 was assessed by preincubating Raji cells washed in FACS buffer with 10 μg/ml of unlabeled mAb for 15 min on ice. Biotinylated FE8 was added at a concentration that had been determined to produce 30% maximal staining for an additional 15 min. After incubation with cychrome 5 PE-labeled streptavidin (Sigma), immunofluorescence was measured by flow cytometry.

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PBL (5 × 10^5/well) or resting tonsil B cells (2 × 10^5/well) were transferred in 50 μl of complete RPMI 1640 medium into 96-well plates. Fifty microliters of mAb diluted in complete medium was added to each well, and the cells were incubated at 37°C with 5% CO2, room atmosphere for 15 min. Then, 150 μl of B95-8 cell culture supernatant or Raji cell supernatant was added and incubated for an additional 90 min at 37°C with a 5% CO2 room atmosphere. Two hundred microliters of culture fluid per well was used.
then replaced by 200 μl of complete medium containing 0.2 μg/ml cyclosporin A, and incubation was continued for 10 days. Finally, two independent investigators recorded the number of outgrowing cell clusters by light microscopy. After adding 1 μCi of [3H]thymidine/well for 24 h, cells were harvested onto glass-fiber filters, and incorporated radioactivity was measured by liquid scintillation.

**Screening of a CR2-derived peptide library with mAb FE8**

A library of 6-mer peptides (five overlapping positions) spanning SCR1 and -2 (amino acids I21-P151 of CR2 (45), i.e., residues 1–131 of the mature CR2 protein) was prepared by automated spot synthesis (46) using a spot synthesizer (Abimed, Langenfeld, Germany) at Jerini Bio-tools (Berlin, Germany). Peptides were C-terminally attached to cellulose via a (β-Ala)2 spacer. After prewashing the membranes with methanol and three times with TBS (31 mM Tris-HCl (pH 7.6), 170 mM NaCl, and 6.4 mM KCl) at room temperature, nonspecific binding was blocked by incubation with B-TBS (TBS containing 0.05% Tween 20, 5% sucrose, and 10% blocking solution (Cambridge Research Biochemicals, Cambridge, U.K.)) for 1 h, followed by incubation with mAb FE8 at 5 μg/ml in B-TBS for 3 h at room temperature. After washing three times for 10 min each time with B-TBS plus 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated rabbit anti-mouse Ab at 1 μg/ml of B-TBS for 2 h. After three washing steps, chemiluminescence signals were obtained using BM chemiluminescence blotting substrate (Boehringer Mannheim, Mannheim, Germany).

**Computer modeling of SCR1 and -2 of CR2**

The three-dimensional structure of SCR1 and -2 was assessed by homology modeling with the Composer model of the Sybyl software suite version 6.3 (Tripos Associates, St. Louis, MO) on a Silicon Graphics Indigo Workstation. A nuclear magnetic resonance-derived structure of SCR15 of complement factor H (Brookhaven database, code no. 1HFH) (17) was used as a template for SCR1 and SCR2. After sequence alignment, the program identified groups of topologically equivalent residues and generated a framework for the CR2 model based on structurally conserved regions. Then, the variable regions were inserted as loops and fitted with matching loops from the Sybyl protein database. Energy minimization to a convergent conformation of 0.05 Cal/(mol × Å) was conducted with Tripos force fields. The final structures were visualized using the RasMol program, version 2.6 (courtesy of Dr. R. Sayle, Glaxo, Middlesex, U.K.).

**Results**

**mAb FE8 recognizes and blocks the C3dg binding site of CR2**

Much of the previous work characterizing the C3dg/EBV binding site on CR2 has been accomplished using mAb OKB7 (19), and to date, OKB7 has been the only tool available for a structural characterization of this site. However, when we tested the inhibitory effect of OKB7 on C3dg binding under several experimental conditions, OKB7 was partly ineffective. With C3d-coated FITC-labeled agarose microbeads as a ligand for CR2 on Raji B lymphoblastoid cells, the maximal inhibition of bead attachment reached with OKB7 was 30% (Fig. 1). Furthermore, we have previously observed that OKB7 blocked CR2-dependent activation of the alternative pathway only after a second Ab (anti-mouse Ig) was included (33). This together with the current unavailability of OKB7 from commercial sources led us to establish additional C3dg-blocking mAbs using rsCR2.1–4, a soluble fragment of CR2 containing the first four SCRs expressed in the baculovirus system (34), as Ag. Of the resulting 65 hybridomas that reacted with rsCR2.1–4, one clone, FE8 (IgG1 κ), was selected by screening for inhibitory effects on the binding of C3d-coated microbeads to Raji cells. As shown in Figure 1, such an effect was seen only with mAbs FE8 and OKB7, but not with HB5 or any other hybridoma supernatant from these fusions. Although FE8 and OKB7 had similar dose-response characteristics, only FE8 completely abrogated binding of this multiply C3d-coated ligand. Uniquely, FE8 was also able to dissociate C3d microbeads that were already bound to CR2 (data not shown).

To address whether the qualitatively similar effects of mAbs FE8 and OKB7 would be reflected by an overlap of their epitopes, competition for CR2 binding was tested. OKB7 inhibited FE8-dependent fluorescent staining to 45% when added at a 100-fold molar excess (Table I). Thus, the epitopes are not identical, but are in close proximity or overlapping to some extent.

**mAb FE8 dissociates bound C3 fragments from CR2**

Next we investigated whether FE8 is able to dissociate CR2-bound C3 fragments from CR2. When Raji cells were preincubated with 20 μg/ml C3dg, FE8, but not OKB7, added afterward led to complete dissociation of C3dg from CR2 (Fig. 2). Whether FE8 was incubated at 25 or 4°C did not alter its binding or the potential to dissociate soluble C3dg. In contrast, OKB7 bound to CR2 in comparable amounts under both conditions, but was clearly less effective to dissociate soluble C3dg at 25°C (Fig. 2A, lower part) than at 4°C (Fig. 2A, upper part). In a two-color FACS analysis, the correlation between mAb and C3dg is most pronounced for OKB7 incubated at 25°C (Fig. 2B, box 4) which means that OKB7 and C3dg can simultaneously bind to CR2. Thus, the C3dg-blocking effect of OKB7 may to some extent be due to steric hindrance, which is more easily overcome at higher temperatures, whereas this is not the case for FE8.

**Inhibition of in vitro EBV infection of B lymphocytes**

As mAb FE8 interfered efficiently with binding of the physiologic CR2 ligand C3dg, we tested the effect of FE8 on attachment of EBV to CR2 on peripheral blood B cells and purified resting tonsil B cells. Outgrowth of B cell clones transformed by the B95-8 strain of EBV was quantified by [3H]thymidine incorporation. Preincubation of PBL isolated from six healthy donors with increasing

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<th>Staining mAb*</th>
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<tr>
<td>IIC5 (ctrl.)</td>
<td>(none)</td>
<td>3.3</td>
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<tr>
<td>FE8</td>
<td>TIB 191 (ctrl.)</td>
<td>44.5</td>
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*Unconjugated mAb at 100-fold molar excess.
concentrations of either FE8 or OKB7 reduced EBV infectivity to background levels (Fig. 3A), which were also observed with EBV preincubated with mAb 72A1 or without EBV (not shown). In contrast to C3dg blockade, the effects of FE8 and OKB7 on in vitro EBV infection did not significantly differ from each other. This was consistently observed for all the donors tested. Isotype control mAbs had no inhibitory effect on the number of outgrowing clones (not shown) or [3 H]thymidine incorporation (Fig. 3A).

The same influence was observed in experiments with resting B cells purified from tonsils (Fig. 3B). A 1:1 mixture of FE8 and OKB7 was equally effective as each of the mAbs alone. Proliferation of resting tonsil B cells incubated without EBV ranged from 100 to 200 cpm and was uninfluenced by preincubation with mAbs (not shown).

Epitope mapping for mAb FE8 by screening a cellulose-bound peptide library

In preliminary experiments we observed that FE8 did not react with rsCR2.1–4 coated to ELISA plates under standard mild alkaline conditions or with either rsCR2.1–4 or Raji cell CR2 in Western blots (either reducing or nonreducing conditions). In contrast, FE8 was successfully used for immunoprecipitation of the 145-kDa CR2 molecule from Raji cell lysates or for FACS analysis of all CR2-expressing cell lines that were investigated, including the Raji, Ramos, and Daudi cell lines (data not shown).

This suggested that FE8 probably recognizes a discontinuous epitope. To define the amino acid residues contributing to this epitope, a library of overlapping hexapeptides covering the sequences of SCR1 and SCR2 (Fig. 4B) was synthesized and assayed for binding of FE8 by the peptide-scanning technique as described in Materials and Methods. Several spots were clearly recognized (Fig. 4A). Peptides 63 and 64 (EYFNKY and YFNKYS) reacted most intensively. Together with the more weakly stained peptide...
65 (FNKYSS) they comprise the eight amino acids between the fourth Cys of SCR1 and the first Cys of SCR2 and thus the entire link region between SCR1 and SCR2. Incorporation of either of the neighboring cysteines C62 and C71 abrogated Ab binding. The third intensively stained spot was observed with peptide 16 (YYS-TPI), which directly follows the sequence 8-PILNGRIS-15 that has been described by others as important for binding of OKB7 and iC3b (28, 32). Peptides 8 and 9, which span that sequence, were not stained; peptide 11 (NGRISY) reacted faintly. A weak reaction was also observed with peptides 87, 88, and 105. Peptide 105 is directly followed in sequence by peptides 110/111, which reacted more clearly.

Three-dimensional model of the FE8 epitope

To investigate whether the five CR2-derived peptides recognized by mAb FE8 may be integrated into a conformational epitope on native CR2, a three-dimensional model was generated. Computer modeling of SCR1 and -2 of CR2 was based on the existing nuclear magnetic resonance-derived structure of factor H SCR15 that could be used as a plausible template due to the high degree of similarity between SCR structures. Furthermore, the positions of disulfide bonds between conserved cysteine residues set a rigid frame for the overall structure that largely limits the degree of freedom of the loops interconnecting them.

As shown in Figure 5, the localization of FE8 binding peptides in this model revealed that the relevant amino acid residues in SCR2 (shown in green, light blue, and dark blue) and the link region EYFNKYS between the SCRs (shown in red) are located on the same side of the molecule and in close proximity to each other. Additionally, the localization of peptide YYSTPI (drawn in magenta) in SCR1 is very compatible with the concept of a conformational epitope encompassing contiguous parts of both SCRs.

Discussion

CR2 fulfills an important bridging function at the intersection of innate host defense and acquired humoral immunity (6). As a receptor for the C3dg molecules covalently deposited on antigenic surfaces, CR2 allows cross-linking of the CR2/CD19/CD81 receptor complex with the BCR, which lowers the threshold for activation of the B cell. The N-terminal pair of SCRs that constitutes this ligand binding site exhibits a 104-fold difference in its affinity for
the C3dg monomer and gp350/220 of EBV. Like CR1 (47), the low affinity of CR2 for the C3 fragment physiologically requires its multiple presence on the antigenic surface, whereas gp350/220 binding is univalent (48). For polymeric C3dg (10–20 mer) prepared by glutaraldehyde cross-linking, the $K_d$ from Raji cell CR2 was 0.7 nM (11).

Most of the work characterizing the ligand binding site of CR2 has employed the mAb OKB7, which blocked rosetting with EAC3d (19) as well as EBV infection (20). Among the many CR2 Abs, mAb AB5 has been reported to block 60% of rosettes formed between Raji cells and C3dg-coated microspheres (49), and mAb MCA-664 (clone B-E5) has been mapped to SCR1–2 (12), although its ligand-blocking potential has not been tested. The reason for this low number of ligand-blocking mAbs might be due to chance or might reflect the possibility that the outermost part of CR2 may be more prone to structural changes than other parts under the conditions used for immunization.

This study aimed at a better understanding of the structural requirements of ligand binding to CR2. The repertoire of existing mAbs useful for this approach was very limited. Additionally, mAb OKB7 was relatively inefficient to inhibit the attachment of C3d-coated particles. Thus, we sought to achieve complete blockade of physiologically C3dg-coated ligands by new mAbs. Although immunization was performed with a recombinant soluble CR2 molecule truncated after SCR4 (34), the yield of clones producing blocking mAbs was very low, and only mAb FE8 exerted a potent inhibitory effect on C3d microbead attachment. The reason for this inefficiency may be that the epitope for C3dg binding is relatively inaccessible or is dependent on a configuration that is easily destroyed. The sensitivity of the FE8 epitope to even mild denaturing conditions would argue in favor of this hypothesis. Likewise, steric hindrance would be expected to occur with some of the above nonblocking mAbs if the C3dg site were exposed.

The results presented here suggest that the new mAb FE8 matches very well with this site, as can be concluded from the experiments that showed a high efficiency in blockade of C3dg under various experimental conditions (Figs. 1 and 2). Although OKB7 fully inhibited rosetting with EAC3d or EAIc3b in our hands, blocking of particles with multiple C3d residues covalently attached in the physiologic orientation could not be achieved. Interestingly, OKB7 was also less active on dissociation of CR2-bound soluble C3dg at room temperature compared with that at 4°C, which may be explained by a substantial portion of steric hindrance contributing to the inhibitory potential. In accordance with this finding, FE8, but not OKB7, completely blocked CR2-dependent activation of the alternative complement pathway, suggesting that attachment of the initial C3 convertase iC3bBbP is abrogated only via the FE8 epitope (M. G. Schwendinger, L. Kacani, A. Sprinzl, M. Wurm, M. P. Dierich, and W. M. Prodinger, manuscript in preparation).

As would be suggested by its C3d inhibitory potential, mAb FE8 interfered with attachment of gp350/220 from EBV. Interestingly, the effects on in vitro EBV infection were less divergent between OKB7 and FE8. Both showed similar dose-response curves and in the fully effective concentrations suppressed EBV-infection as completely as did mAb 72A1 (35) directed against gp350/220. The difference in the inhibitory Ab concentrations seen between B lymphocytes prepared from peripheral blood or from tonsils was most likely due to a difference in the amount of B cells present.

The regions in SCR1 and -2 that are crucial for ligand binding to CR2 have been investigated by others using a series of many human/mouse chimeras or peptide inhibition of EAC rosetting. Martin et al. suggested that binding of gp350/220 to the CR2 N terminus is due to the overall conformation of SCR1 and -2. None of the inter-Cys sequences abrogated EBV binding when individually replaced with the corresponding sequence from mouse CR2 (31). Our finding that two mAbs binding to different epitopes similarly interfere with EBV infection is consistent with this report of Martin et al. Their later report of two amino acid substitutions (S15P and Y68T) in mouse CR2 that make EBV infection possible (32) need not be contradictory, as both substitutions entail marked structural alterations, either by the loss of a proline residue or by the loss of a consensus glycosylation site.

The amino acid residues that contribute to binding of C3b-derived ligands to CR2 have been addressed by Molina et al. by inhibition of EAIc3b rosette formation with peptides (28). They suggested that the sequences 10-LNGRIS-15 in SCR 1 and 84-GSTPRHGDSVTFA-97 in SCR 2 are critical, with a potential further contribution of peptide 99-KTNFSMNKNSVW-111. These findings are supported by the analogy to mouse CR2: mAbs 7G6 and 4E3 that independently block C3dg binding (30) recognize the parts of mouse CR2 that correspond to these sequences from human CR2 (28, 31).

Both the P8-S15 sequence in SCR1 and the inter-SCR region E63-S70 have been determined as crucial for binding of mAb OKB7 by independent investigations with human/mouse chimeras (31, 50). However, the structural impact of an additional consensus N-glycosylation site in the link region of mouse CR2 may alternatively explain the importance of the inter-SCR region for OKB7 and EBV binding regardless of the neighboring residues. Absence of this carbohydrate residue was required but not sufficient for OKB7 binding, which occurred only when the sequence 3-GSPPP PILGRISY-16 was also present (31). Whether introduction of the N-glycosylation site into human CR2 would abrogate EBV and/or OKB7 binding has not been tested.

In the present study we have employed peptide scanning to investigate the epitope of the new mAb FE8 in more detail. Interestingly, peptides for the inter-SCR sequence E63-S69 were most intensively stained by mAb FE8. Thus, although E63-S69 has not been shown to inhibit EAIc3b rosetting as a soluble peptide (28), the recognition of this link region could be demonstrated for mAb FE8 and may be crucial for its C3dg-blocking effect. Other CR2 regions that comprise residues probably interacting with FE8 were Y16-I21, P87-D92, and N105-N116. Y16-I21 follows immediately downstream the sequence 8-PILNGRIS-15 that is important for interaction with OKB7 and iC3b. In the three-dimensional model based on SCR15 and -16 of factor H (17), the stretch P8-I21 may be seen as a knuckle protruding into the recess between SCR1 and -2, also contributing to the boundaries of this region. Whether introduction of the N-glycosylation site into human CR2 would abrogate EBV and/or OKB7 binding has not been tested.
Acknowledgments

Helmut Gruberhofer and Beate Michel contributed excellent technical assistance to this work. We are grateful to Johannes Möst for sharing PBL preparations, and to B. M. Rode for the possibility of working with Sybyl software.

References