

# Epitope Mapping Using the X-Ray Crystallographic Structure of Complement Receptor Type 2 (CR2)/CD21: Identification of a Highly Inhibitory Monoclonal Antibody That Directly Recognizes the CR2-C3d Interface<sup>1</sup>

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Complement receptor type 2 (CR2)/CD21 is a B lymphocyte cell membrane C3d/iC3b receptor that plays a central role in the immune response. Human CR2 is also the receptor for the EBV viral membrane glycoprotein gp350/220. Both C3d and gp350/220 bind CR2 within the first two of 15–16 repetitive domains that have been designated short consensus/complement repeats. Many mAbs react with human CR2; however, only one currently available mAb is known to block both C3d/iC3b and gp350/220 binding. We have used a recombinant form of human CR2 containing the short consensus/complement repeat 1-2 ligand-binding fragment to immunize *Cr2*<sup>-/-</sup> mice. Following fusion, we identified and further characterized four new anti-CR2 mAbs that recognize this fragment. Three of these inhibited binding of CR2 to C3d and gp350/220 in different forms. We have determined the relative inhibitory ability of the four mAbs to block ligand binding, and we have used overlapping peptide-based approaches to identify linear epitopes recognized by the inhibitory mAbs. Placement of these epitopes on the recently solved crystal structure of the CR2-C3d complex reveals that each inhibitory mAb recognizes a site either within or adjacent to the CR2-C3d contact site. One new mAb, designated 171, blocks CR2 receptor-ligand interactions with the greatest efficiency and recognizes a portion of the C3d contact site on CR2. Thus, we have created an anti-human CR2 mAb that blocks the C3d ligand by direct contact with its interaction site, and we have provided confirmatory evidence that the C3d binding site seen in its crystal structure exists in solution. *The Journal of Immunology*, 2001, 167: 5758–5766.

**H**uman complement receptor type 2 (CR2)<sup>3</sup>/CD21 is an ~145-kDa membrane glycoprotein expressed on mature B cells, follicular dendritic cells (FDC), epithelial cells, mast cells, thymocytes, and a subpopulation of peripheral  $\alpha\beta$  T lymphocytes (reviewed in Refs. 1–3). The primary ligand for CR2 is the C3d activation fragment of the complement component C3 (4). In addition to C3d, the larger C3dg and iC3b fragments of C3 also bind CR2 with high affinity (5, 6). CR2 is also a receptor for EBV by binding the surface glycoprotein gp350/220 (7, 8) and serves as a receptor for CD23 (9). CR2 is a member of a structural

family of C3/C4 receptor and regulatory proteins designated the regulators of complement activation (10) and contains 15 or 16 of the domains that define this short consensus/complement repeat (SCR) superfamily.

As a C3 receptor, CR2 has been shown to play a critical role in humoral immune responses to T-dependent Ags (11–13) and thereby acts as a bridge between innate and adaptive immunity (14). Expression of CR2 on both B cells (11–13) as well as FDC (13) is critical to its role in T-dependent Ag responses. The primary role of CR2 on B cells is to lower the threshold for activation of Ag-specific B cells by coligating the Ag-specific B cell receptor with the CR2/CD19/CD81 signaling complex (3, 15). This immune enhancing activity of CR2 has been used to create highly immunogenic C3d-bound Ags that generate robust humoral responses even in the absence of adjuvant (16). CR2 can also mediate uptake and presentation of Ags to specific T cells (17, 18). As a receptor for CD23, CR2 promotes B cell class switching and production of IgE isotype Abs (9), rescues germinal center B cells from apoptosis (19), and enhances T-B cell-cell adhesive interactions (20).

As the EBV receptor, CR2 mediates infection and activation of B cells by this virus (21). EBV is an important human pathogen that causes infectious mononucleosis and is also associated with an increasing number of cancers including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, and T cell lymphoma, as well as gastric and breast cancer (reviewed in Ref. 22). EBV also causes an aggressive lymphoproliferative disorder in immunocompromised patients, such as individuals with severe HIV-1 infection or those taking immunosuppressive drugs following

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<sup>3</sup> Abbreviations used in this paper: CR2, complement receptor type 2; CR1, complement receptor type 1; FDC, follicular dendritic cell; SCR, short consensus/complement repeat; E, erythrocyte; SLE, systemic lupus erythematosus.

solid organ or bone marrow transplantation. In addition, infection with EBV has been associated with autoimmune diseases including systemic lupus erythematosus (SLE) (23), rheumatoid arthritis (24), and multiple sclerosis (25).

Decreased expression of CR2 on B cells was found in patients with SLE and thus was initially implicated in the dysregulation of tolerance to self Ags in this illness (26, 27). In murine models of SLE, decreased expression of CR2 and the alternatively spliced complement receptor type 1 (CR1) has been found (28). In addition, increased levels of autoantibodies and decreased energy indicative of a loss of self tolerance are present when the *Cr2* gene encoding CR2 and CR1 in mice has been inactivated by gene targeting and then crossed into the B6/*lpr* strain (29).

On FDC, CR2 serves as a receptor for C3-bound immune complexes and can therefore mediate retention of Ags for prolonged periods (30). One particularly relevant outcome of this activity is the retention on FDC of highly infectious HIV-1 present as C3-bound immune complexes (31, 32) that can be released from germinal centers *in vitro* by inhibition of C3-CR2 binding (33). CR2-expressing thymocytes and peripheral T cells can also be infected with HIV-1 immune complexes containing C3 ligands through this receptor (34, 35). In addition, freshly isolated peripheral blood and lymph node B cells from HIV-infected patients who are chronically viremic have also been shown to efficiently transfer CR2-bound immune complexes containing HIV-1 from their surface and infect naive T cells using a CD4-dependent mechanism (36).

The availability of well-characterized and highly inhibitory mAbs directed against the SCR 1-2 ligand-binding fragment of CR2 would provide an important tool to help determine structure-function relationships as well as further study *in vivo* roles of CR2 and perhaps allow the therapeutic targeting of this receptor. However, with the exception of one recently described mAb, FE8 (37), no currently available mAb exhibits significant inhibition of C3d and EBV binding to CR2. One previously characterized mAb, designated OKB7, is no longer commercially available. To create inhibitory mAbs, we have used *Cr2*<sup>-/-</sup> mice and immunized them with a fragment of recombinant human CR2 containing only the SCR 1-2 ligand-binding fragment. Using the finding that strong adjuvants can overcome in part the humoral immune defect in these mice (38), we generated high-titer antiserum and then four new mAbs, termed 171, 1048, 994, and 629, each of which reacts with the SCR 1-2 fragment. Three of the four mAbs (171, 1048, and 994) block C3d and gp350/220 ligand binding to CR2. The epitopes of these inhibitory mAbs, as well as those previously defined for mAb FE8 (37) and mAb OKB7 (39), have been placed on the three-dimensional structure of CR2 that we have recently solved in complex with its C3d ligand (40). The most highly inhibitory mAb, 171, recognizes an epitope that substantially overlaps with the interface between CR2 and C3d. Thus, we have created the first structure-guided epitope map of CR2, and in this process we have also obtained additional confirmatory evidence that the x-ray crystallographic structure of the CR2-C3d complex exists in solution.

## Materials and Methods

### Production of recombinant CR2

Two previously described forms of human CR2 were used for these studies. Briefly, a soluble recombinant form of human CR2 containing only SCR 1-2 was produced in *Pichia pastoris* using the *pPICZα* expression plasmid transfected into the wild-type X33 strain of *Pichia* (41). Protein production was induced by addition of methanol to the minimal fermentation medium. Recombinant protein was first passed over SP-Sepharose, then deglycosylated with Endo-H<sub>f</sub> to remove all carbohydrates except *N*-acetyl glucosamine attached to N residues, and repurified on an SP-HiTrap column (Amersham Pharmacia Biotech, Piscataway, NJ). Deglycosylated

SCR 1-2 was further purified using a Superdex-75 (Amersham Pharmacia Biotech) gel filtration column. The second form of human CR2 was a soluble, full-length SCR 1-15 form of CR2 produced in a baculoviral expression system (42). To purify this, culture supernatants containing CR2 SCR 1-15 were passed over Q-Sepharose (Amersham Pharmacia Biotech) anion exchange columns followed by gel filtration using a Superose-6 column (Amersham Pharmacia Biotech).

### Immunization of *Cr2*<sup>-/-</sup> mice, and generation and initial screening of anti-CR2 hybridomas

*Cr2*<sup>-/-</sup> mice (13) that had been back-crossed for seven generations to the C57BL/6 background were immunized with 50 μg of human CR2 SCR 1-2 in CFA, followed by three more monthly immunizations with 50 μg of CR2 SCR 1-2 in IFA. Sera from immunized mice were tested using a direct ELISA where CR2 SCR 1-15 was immobilized to the solid phase and Ab binding was detected using HRP-conjugated γ chain-specific goat anti-mouse IgG (Cappel, Burlingame, CA). One week before fusion, the mouse with the most robust response was immunized *i.v.* with 50 μg of CR2 SCR 1-2 in normal saline. Spleen cells from this immunized mouse were isolated and fused with Fox-NY nonsecreting myeloma cells in a standard hybridoma fusion protocol in the University of Colorado Cancer Center Tissue Culture Core Facility (Denver, CO). Subcloned cells from this fusion were screened for specific mAb production. Of the >2000 wells screened, 30 wells were initially positive. Based on levels of reactivity, 10 of these were initially subcloned and optimized for expression and functional testing. Of these 10, four possessed reproducible ability to recognize human CR2 in different forms and warranted further study. Each of these four mAbs was shown to be the IgG1 isotype by both isotype-specific ELISA and isotype-specific Western blots. For all studies performed herein, mAbs were used that had been purified by passage of exhausted culture supernatants over protein G-Sepharose resin followed by low pH elution, neutralization, and dialysis into PBS, pH 7.4.

### Flow cytometry

Flow cytometric experiments were performed using K562 human erythroleukemia cells that were transfected with full-length human CR2 as previously described (43). In each case, 5 × 10<sup>5</sup> human CR2-transfected or antisense construct-transfected K562 cells were incubated with either control IgG1 mAb MOPC-31, well-characterized anti-CR2 mAbs HB-5 and OKB7 (39, 43, 44), or the four anti-human CR2 mAbs described in this report at 1 μg/ml on ice. Following washing, the reactivity of the mAbs was detected using biotin-conjugated anti-mouse IgG (BD Pharmingen, San Diego, CA) followed by PE-conjugated streptavidin and analysis by single-color flow cytometry in the University of Colorado Cancer Center Flow Cytometry Core Facility (Denver, CO).

### Competitive ELISA

As one measure of the ability of the new anti-human CR2 mAbs to inhibit ligand binding, competition ELISA was performed. The CR2 ligands, either C3dg produced and purified from aged human serum as described (43), or a 70-kDa fragment of EBV gp350/220 produced in baculovirus and containing the CR2 binding site (45), were immobilized to ELISA plates at a concentration of 10 μg/ml. Plates were blocked with PBS and 1% BSA, washed with PBS and 0.05% Tween 20 (pH 7.4), and then incubated with CR2 (SCR 1-15 from baculovirus) that had been pretreated for 40 min with varying concentrations of control or specific anti-CR2 mAbs. Following washing, bound CR2 was detected by incubation of wells with rabbit polyclonal anti-CR2 Ab, followed by washing again, treatment with HRP-conjugated goat anti-rabbit Ab IgG (Bio-Rad, Hercules, CA) and development. In a second type of analysis to compare the binding sites of mAbs and ligand on CR2, wells that had been treated exactly as above, but without preincubation of the CR2 SCR 1-15 with anti-CR2 mAbs before incubation with ligand-bound plates, were then treated with varying dilutions of the same mAbs for 40 min. The presence of individual anti-CR2 mAbs in the reaction well originally containing ligand and CR2 SCR 1-15 was then detected following washing using HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

### Inhibition of erythrocyte rosette formation

Human CR2-expressing K562 cells were used in triplicate in a rosette assay using SRBC coated with human C3d (EAC3d) obtained from the Complement Reference Laboratory at the National Jewish Medical and Research Center (Denver, CO; P. Giclas, director). These erythrocytes (Es) contain C3 in the form of C3dg where conversion of iC3b to C3dg is monitored by loss of rosette formation with indicator neutrophils. EAC3d were then mixed for 20 min at 37°C with human CR2-expressing K562

cells that had been either untreated or preincubated for 20 min at room temperature with control or anti-CR2 SCR 1-2 mAbs. The percentage of inhibition of rosette formation was calculated based upon the untreated rosette formation values compared with those obtained using K562 cells pretreated with the anti-human CR2 mAbs.

#### *Inhibition of EBV-induced lymphoproliferation*

Inhibition of EBV-induced lymphoproliferation was measured as previously described (37). PBMC ( $5 \times 10^5$  per well) isolated from normal human donors were preincubated with control or anti-CR2 SCR 1-2 mAbs at varying concentrations for 60 min at 37°C. EBV containing culture supernatants from the B95-8 marmoset cell line were then added along with 10  $\mu$ g/ml cyclosporin A. These cultures were incubated for 10 days at 37°C in a CO<sub>2</sub> incubator. During the last 16 h of culture, 10  $\mu$ Ci of [<sup>3</sup>H]thymidine was added to each well. The cultures were harvested onto glass filter mats and the amount of proliferation as measured by [<sup>3</sup>H]thymidine incorporation was assessed by liquid scintillation counting. Results were expressed as the percentage of inhibition of EBV-induced proliferation in the mAb-pretreated PBMC cultures compared with the untreated PBMC cultures.

#### *Epitope mapping of CR2 mAbs using mimotope multipin analysis*

Linear mimotopes of human CR2 SCR 1-2 were produced using the multipin analysis technique (Mimotopes, San Diego, CA). All possible 8-mer peptides of CR2 SCR 1-2, each offset by one amino acid, were synthesized onto the solid-phase supports arranged in the format of a 96-well ELISA plate. In addition, overlapping 5-mer, 6-mer, and 7-mer peptides from two previously identified CR2 SCR 1-2-derived peptides that blocked iC3b-dependent erythrocyte rosette formation with CR2 (LNGRIS and GST-PYRHGDSVTFA) (46) were also synthesized on the solid-phase supports. All peptides were then tested by modified ELISA as previously described (47) with each of the control or anti-human CR2 SCR 1-2 mAbs. Binding of mAbs to individual peptides was then determined using alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG Ab.

#### *HIV displacement from B cells by CR2 mAbs*

PBMC-derived B cells were isolated from HIV-infected chronically viremic patients and subjected to HIV-displacing assays as previously described (36). In brief, the isolated B cells were preincubated under various conditions with the nondisplacing anti-CR2 mAb BL13 (Beckman Coulter, Fullerton, CA) or the anti-CR2 mAbs 171, 1048, and FE8. mAb FE8 was provided by Dr. W. M. Prodinger (University of Innsbruck, Innsbruck, Austria) (37). HIV is presumably bound to CR2 by a mixture of C3d, C3dg, and iC3b fragments covalently attached to the viral particles by *in vivo* immune recognition and cleavage mechanisms (36). The B cells were then cocultured with anti-CD3-stimulated HIV-negative PBMCs depleted of CD8-positive T cells (indicator T cells). Productive replication of HIV was evaluated by ELISA determination of HIV-1 p24 (Beckman Coulter).

#### *Epitope mapping of mAbs on CR2 three-dimensional structure*

The CR2 peptides that have been shown to react with mAbs were color-coded on the sequence of the CR2 SCR 1-2 crystal structure. This color was then mapped to the surface of the CR2 crystal structure using the program GRASP (A. Nichols, Columbia University, New York, NY) (48). For comparison, the C3d-binding interface of CR2 SCR 1-2 was also color-mapped in this way.

## Results

### *Generation of novel mAbs in Cr2<sup>-/-</sup> mice that recognize human CR2*

Despite an extensive analysis of available mAbs to human CR2, only two, FE8 and OKB7, are known to recognize the SCR 1-2 fragment and interfere with ligand binding, and only one, FE8, (37) is currently available. Thus, to bias the results of our experiments and generate an effective immune response focused on the CR2 SCR 1-2 fragment, a recombinant protein containing only this particular fragment of CR2 was used as an immunogen. In addition, a strategy of immunizing Cr2<sup>-/-</sup> mice was used so that this fragment would be seen as foreign. Using a strong adjuvant, an approach shown to ameliorate several of the humoral immune defects in Cr2<sup>-/-</sup> mice (38) allowed us to generate high-titer anti-human CR2 antiserum in these mice.

In addition to using the CR2 SCR 1-2 fragment produced in *Pichia* as the specific immunogen, human CR2 SCR 1-15 produced in a baculovirus expression system was used during the hybridoma screening process. This enabled us to eliminate hybridomas that detected mAbs specific for other determinants on the Ag, such as the *N*-acetyl glucosamine remaining on the immunogen in association with the peptide backbone, or the epitope tag. Using this strategy, we were able to create four new mAbs, 171, 1048, 994, and 629, that reacted with the SCR 1-2 ligand binding fragment as well as wild-type CR2 (Table I).

#### *Immunoreactivity of new anti-human CR2 mAbs*

To determine whether the four new mAbs would recognize SDS denatured protein, Western blot analysis using nonreducing conditions was performed. Each of the mAbs was found to recognize both full-length human CR2 containing SCR 1-15 as well as SCR 1-2 alone (data not shown). Each of the four mAbs was then tested for its ability to recognize CR2 by flow cytometry of previously described (43) human K562 cells expressing CR2 (Fig. 1). All four readily recognized CR2. Of interest, there was no reactivity with mouse CR2-expressing K562 cells (data not shown).

#### *Effects of mAbs on C3dg and gp350 binding to CR2*

The four new mAbs were then extensively tested to determine their individual abilities to block CR2 ligand-binding activities. Shown in Fig. 2A are results of inhibition studies of soluble CR2 binding to plate-bound C3dg by mAbs. In this analysis, a rank order of inhibition was found in which mAb 171 was the most potent, followed by mAb 1048 and mAb 994, while mAb 629 did not exhibit any inhibitory activity.

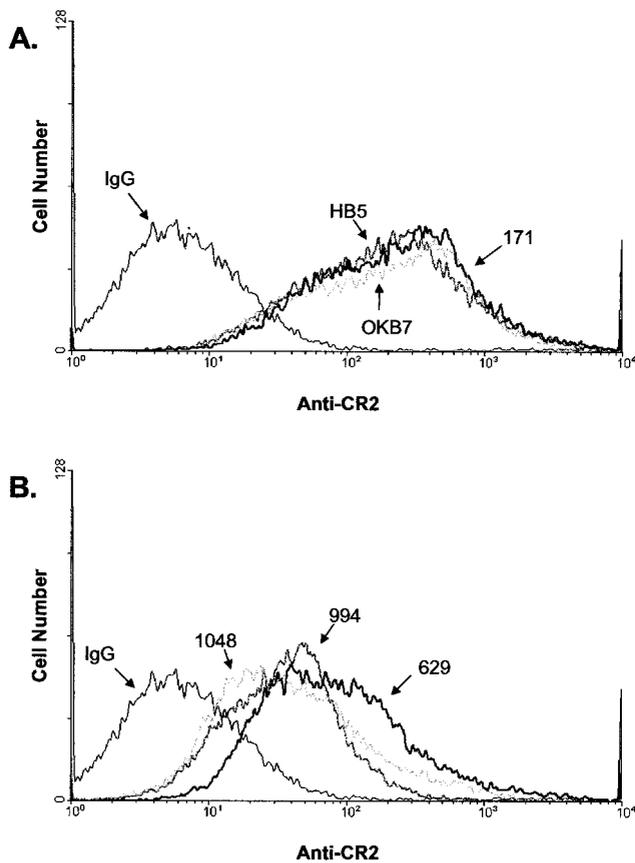
Shown in Fig. 2B are results of a similar analysis in which mAbs were used to block binding of soluble CR2 to the 70-kDa fragment of gp350 (45). The same rank order of inhibition was demonstrated. Together these results suggest that, despite the finding that the C3d and gp350 binding sites on CR2 are not identical (39, 49), they must be related in such a way that the same relative effects of these inhibitory mAbs are seen.

To further extend this analysis, we also determined whether the mAbs could interact with full-length CR2 that had been prebound to each ligand on the plate. In this instance, only mAb HB-5, which reacts with a site within SCR 3-4 (39, 49), and mAb 629 were able to interact with CR2 that is also in contact with either the C3dg (Fig. 2C) or gp350/220 ligand (Fig. 2D). The inability to detect the bound mAbs 171, 1048, or 994 in this setting could be due either to competition of the mAb binding site by the individual ligand or displacement of CR2 from the ligand on the plate. Based on results presented below, we believe the latter is likely to be true.

Table I. Characteristics of mAbs used in these studies<sup>a</sup>

Designation of mAb	Isotype	Minimal SCRs Recognized
New mAbs		
171	IgG1	SCR 1-2
1048	IgG1	SCR 1-2
994	IgG1	SCR 1-2
629	IgG1	SCR 1-2
Previously described mAbs		
HB-5	IgG2a	SCR 3-4
OKB7	IgG2b	SCR 1-2
FE8	IgG1	SCR 1-4

<sup>a</sup> Reported isotypes and minimal number of SCRs required for recognition of CR2. With regard to FE8, it is likely that only SCR 1-2 are required, though this was not directly tested (37).



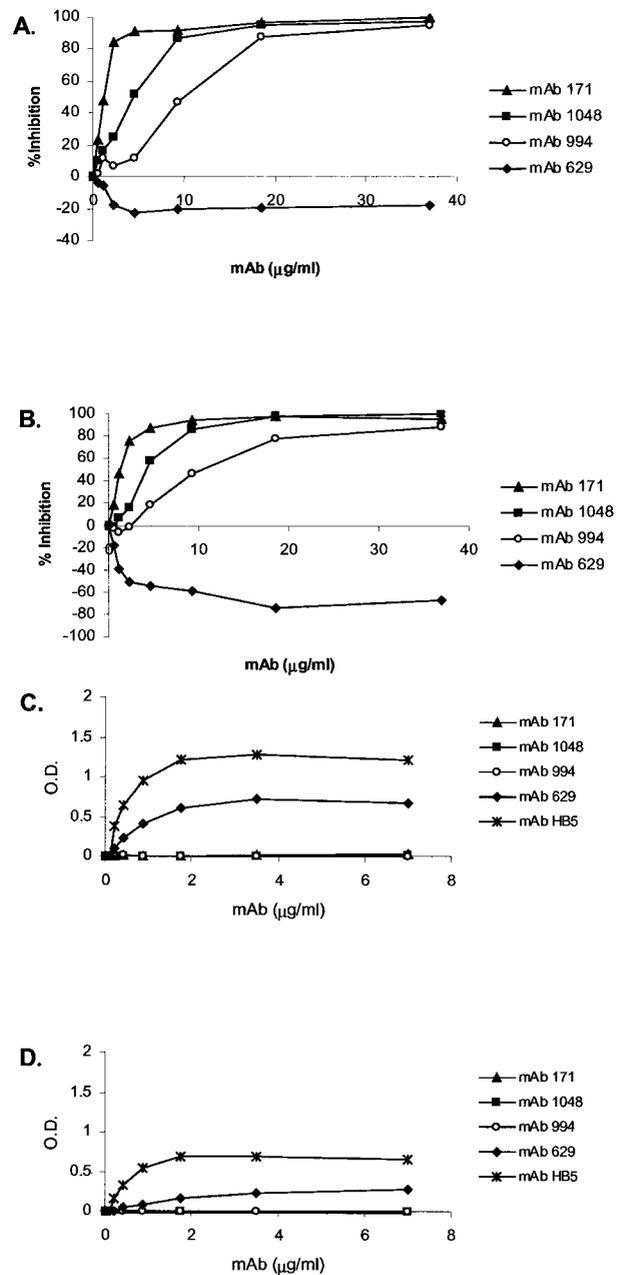
**FIGURE 1.** Flow cytometric analysis using anti-CR2 mAbs of K562 transfectants expressing human CR2. Each of the four new mAbs (171, 1048, 994, and 629) specifically reacts with human CR2-expressing K562 cells, indicating recognition of native conformation. Shown also for comparison are results with mAbs OKB7 and HB-5 (A) and negative control IgG1 mAb (A and B).

To determine whether similar inhibitory effects could be found using natural ligand forms of C3, we performed rosette inhibition studies using Es coated with C3dg (Fig. 3). We also used small remaining amounts of mAb OKB7 to perform a comparative study. Once again, mAb 171 was the most inhibitory mAb, and this was followed by mAb 1048. OKB7, though tested in a limited dose range, appeared to be the next best inhibitor, and this was followed by minimal inhibitory activity of mAbs 994 and 629.

To determine whether the apparent inhibitory effects of mAbs on gp350/220 binding by ELISA were present using a more physiologic model of EBV infection, we used an EBV-dependent primary B cell proliferative assay (37). Fig. 4 demonstrates that at both 2.5 and 10  $\mu\text{g/ml}$ , mAbs 171, 1048, and 994 were highly inhibitory, again in relative rank order, while mAb 629 was not significantly inhibitory. The inhibition is likely due to either direct competition for CR2 binding by virus or to capping of receptor by mAb, as other studies have shown that the mAbs are not directly cytotoxic to human B cell lines in culture (data not shown).

*Relative capacity for displacement of HIV-1-containing immune complexes from CR2 and inhibition of T cell infection*

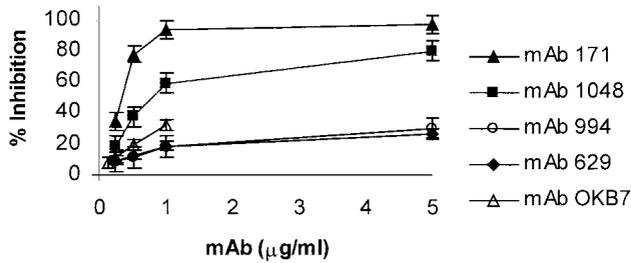
A further analysis of the ability of these new mAbs to block CR2 functions was performed by determining whether each could displace *in vivo* prebound HIV-1-containing immune complexes from the peripheral B cells of patients with active HIV-1 infection (36). In these studies we were also able to directly compare the effects



**FIGURE 2.** Competition ELISA analysis measuring relative ability of mAbs to block ligand binding by CR2. A, Human C3dg is used as the solid-phase ligand and binding of CR2 SCR 1-15 is measured after preincubation with individual mAbs. B, A 70-kDa fragment of EBV gp350/220 is used as the solid-phase ligand and binding of CR2 SCR 1-15 is measured after preincubation with individual mAbs. Results demonstrate that mAbs 171, 1048, and 994 are inhibitory in rank order for each ligand. C and D, The ability of mAbs to interact with wells first coated with ligand and then prebound by CR2 was monitored. The presence of the anti-CR2 mAb in the well following washing was then measured by anti-mouse IgG Ab. C, Wells coated with human C3dg and then incubated with CR2 before addition of anti-CR2 mAb. D, Wells coated with a 70-kDa fragment of EBV gp350/220 and then incubated with CR2 before addition of anti-CR2 mAb. The results demonstrate competition for CR2 binding to both ligands by mAbs 171, 1048, and 994.

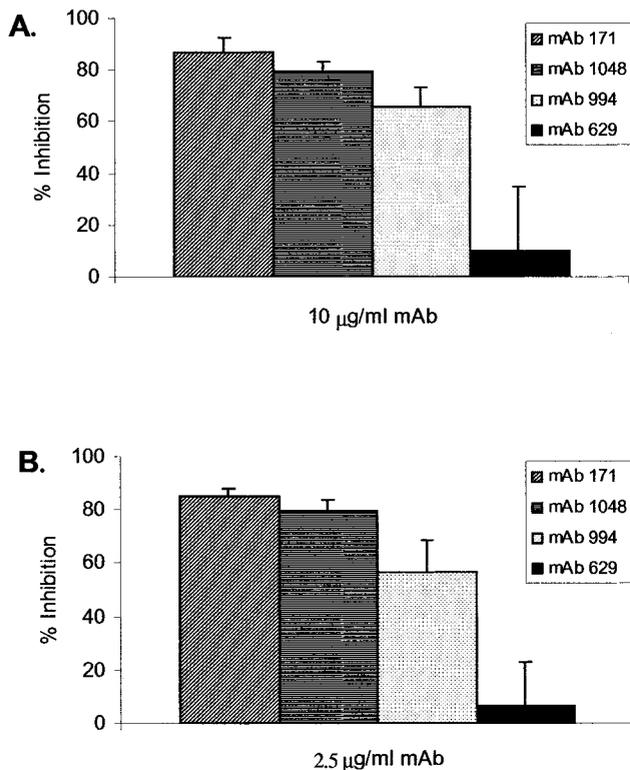
of these mAbs with anti-CR2 mAb FE8, which has been shown previously to be partially effective in this assay (36).

In agreement with the inhibitory activities described in the previous sections, mAb 171 was found to be most effective at displacing HIV-bound C3 fragments from B cells of HIV-infected



**FIGURE 3.** Rosette inhibition analysis using K562 cells expressing CR2 and SRBC coated with C3dg. Shown are means  $\pm$  SD for triplicate measures at each dose. The OKB7 curve was limited by reagent availability. A rank order of inhibition of mAbs 171 and 1048 followed by 994 and 629 is shown.

patients (Fig. 5). In this analysis, mAbs 171, 1048, and FE8 were each able to completely displace C3-complexed HIV after two rounds of preincubation, as compared with the noninhibitory CR2 mAb BL13 (Fig. 5A), while only mAb 171 was able to completely displace the B cell-bound virions after one round of preincubation (Fig. 5B). Furthermore, even when the concentration of Ab and the time of incubation were reduced by half, mAb 171 was still able to completely displace C3-complexed HIV in one round from the B cells while the other two C3-displacing mAbs were ineffective (data not shown). Taken together, these data demonstrate that mAb 171 is a potent inhibitor of the interaction between CR2 and C3 ligand forms found in these naturally occurring immune complexes.



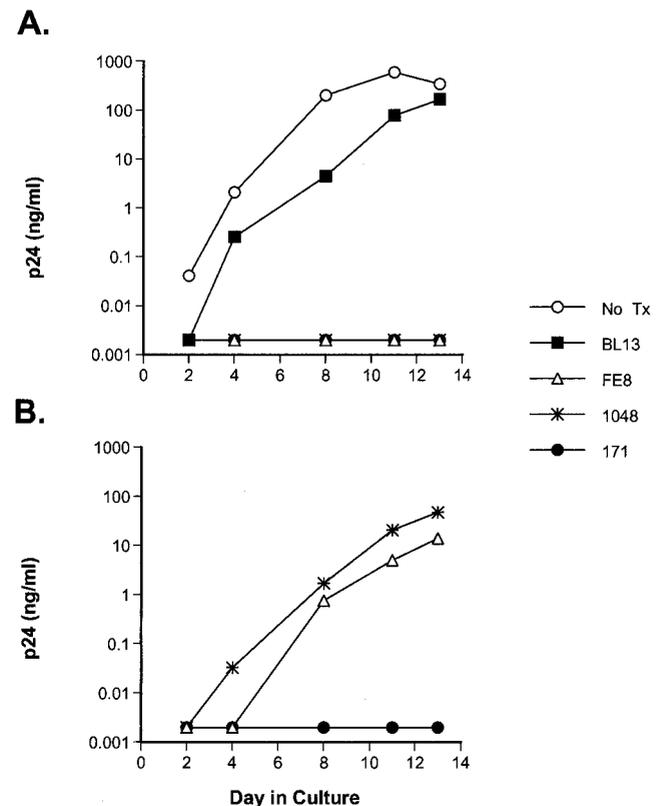
**FIGURE 4.** Inhibition of EBV-induced proliferation of human cyclosporine-treated PBMC cultures as measured by [ $^3$ H]thymidine incorporation during the last 16 h of culture. Each mAb (171, 1048, and 994) that blocks CR2-gp350/220 interactions using purified proteins demonstrated a significant inhibitory effect at both 10 (A) and 2.5  $\mu$ g/ml (B).

### Epitope mapping using mimotope multipin analysis

To determine where each of the mAbs bound to the SCR 1-2 fragment of CR2, we used a technique in which sequential peptides derived from the sequence of this fragment are used as Ag targets. Although this method does not allow the identification of conformational epitopes requiring amino acids from sequentially separate portions of the protein, and does not work with every particular polyclonal or monoclonal Ab, when successful this technique has been used extensively to characterize Ab reactivities with Ag (50) and has been used to identify epitopes on the surface of molecules (51, 52).

In these studies, we used two approaches. First, we synthesized all peptides as 8-mers offset by one amino acid, which is the experimentally determined optimal length for detection of epitopes in an unknown setting (50). In addition, because we had previously identified two sequences from CR2 that when made into peptides would block C3-CR2 interactions (46), we also used an expanded number of peptides of varying lengths from those two regions.

Shown in Fig. 6 are results from this analysis for the three inhibitory mAbs, 171, 1048, and 994. With regard to mAb 171, the sequence TPYRH (peptide 158) was recognized at a level substantially above background. With regard to mAb 1048, the sequence WCQANNMW (peptide 111) was preferentially recognized, and with regard to mAb 994 the neighboring sequence VWCQANNM (peptide 110) was recognized. When mAb OKB7 was tested with this method, no reactivity with any of the peptides was detected

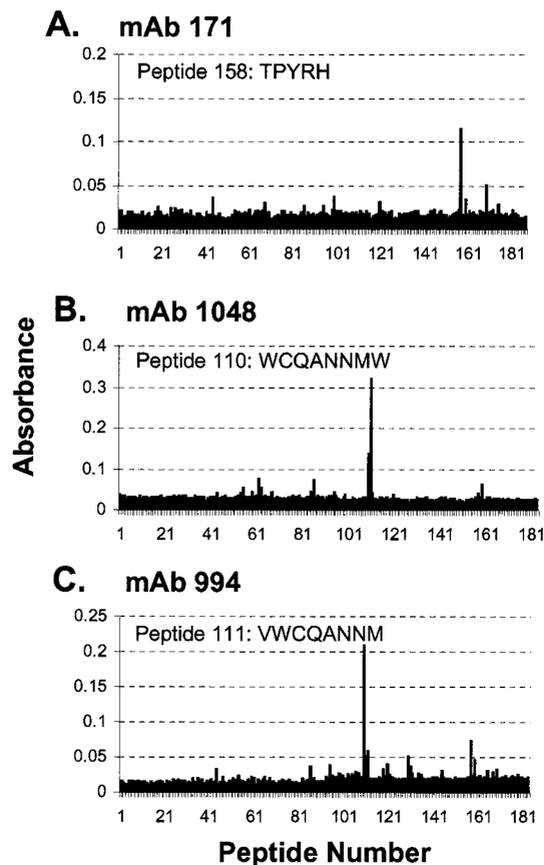


**FIGURE 5.** Relative displacement by mAbs of in vivo prebound HIV-1 immune complexes from B cells of a patient chronically infected with HIV-1. A, B cells were incubated with or without (No Tx) 2  $\mu$ g/ml anti-CR2 mAbs for 60 min at room temperature, washed once, incubated a second time with 2  $\mu$ g/ml mAb for 30 min at room temperature, washed twice, and cocultured with indicator T cells. B, B cells were incubated with 2  $\mu$ g/ml anti-CR2 mAbs for 60 min at room temperature, washed twice, and cocultured with indicator T cells. Shown are representative results of one of a total of four HIV-infected patients tested.

(data not shown). Previous analysis of mAb FE8 using a similar strategy, but with peptides displayed on cellulose membranes rather than on pins, revealed five separate linear sequences recognized by this mAb (see Fig. 7).

#### Modeling of mAb interaction sites based on the CR2-C3d crystal structure

To determine where these mAbs interacted with CR2 in its native state, we used our recently determined crystal structure of the CR2-C3d complex (40). In this structure, C3d primarily interacts with the amino acid sequence YKIRGSTP within SCR 2 of CR2. Of interest, when reactive peptides were overlaid onto the CR2 structure, the first two residues in the epitope for the most inhibitory mAb in our analyses, mAb 171, corresponded to the last two residues in this CR2 contact site with C3d (Fig. 7, A–C). Specifically, within the CR2 sequence 87-TPYRH-91, T87 is at the CR2-C3d interface and forms a hydrogen bond with a main-chain carbonyl group of C3d, while P88 also packs with P69 of C3d (Fig. 7A). YRH are then immediately flanking the interface. In contrast, the overlapping epitopes for mAbs 1048 and 994 are not found within the interface but rather are immediately adjacent to it, albeit in an area of SCR 2 that is pointing away from the interface (Fig. 7C). Therefore, the effects of these two inhibitory mAbs are likely



**FIGURE 6.** Sequential solid phase epitope mapping of inhibitory anti-human CR2 mAbs 171, 1048, and 994. Solid-phase supports containing synthetic 8-mer peptides spanning human CR2 SCR 1-2 using an offset of one amino acid as well as shorter peptides in regions of previously determined candidate C3d binding sites (46) were screened for reactivity with various inhibitory mAbs. Major epitopes recognized (defined as more than three times greater than the average reactivity of isotype-matched mAb with other protein specificity) are peptide 158, TPYRH, for mAb 171; peptide 111, WCQANNMW, for mAb 1048; and peptide 110, VWCQANNM, for mAb 994.

to be either steric or allosteric in nature. With regard to mAb FE8, five distinct linear sequences from CR2 were reported to react with this mAb (37). Its primary reactivity was noted with the 8-aa linker between SCR 1 and SCR 2 (EYFNKYSS), which does not make contact with C3d in the crystal structure (40). The second most intensely recognized peptide sequence was 16-YYSTPI-21, which is within SCR 1, which also does not make contact with C3d. Additional reactivity was noted with peptides 106-NGNKS-111 and 112-WCQANN-117, both within SCR 2 in an area that does not contact C3d. However, a weak reactivity was also noted with peptides encompassing the sequence 88-PYRHGD-93, of which P88 is within the CR2-C3d interface. Fig. 7, D and E, shows the locations of these five FE8-reactive sequences on the surface of CR2.

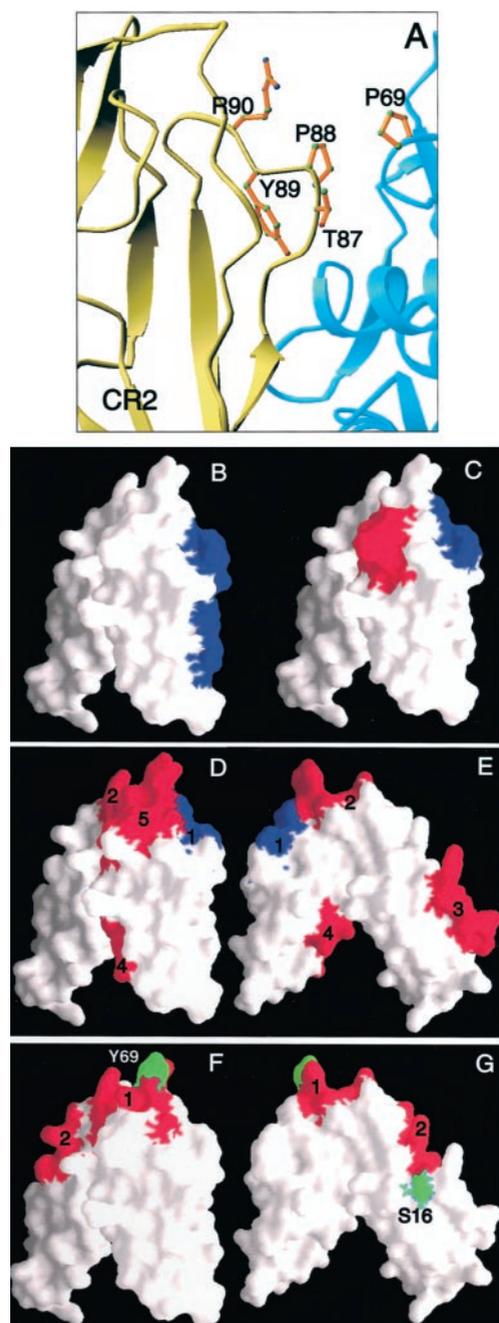
Using gain-of-function analysis and mapping of OKB7 epitopes by mutagenesis, Martin et al. (39) previously identified the amino acids 8-PILNGRIS-15 and the inter-SCR linker 64-EYFNKYSS-71, especially Y69, to be critical for mAb OKB7 binding. From these studies it was not clear whether Y69 itself was a critical residue or whether loss of a glycosylation site in the mouse inter-SCR linker allowed OKB7 binding to the linker region. In addition, two amino acids (P15 and T68), when mutated from mouse to human CR2 sequence by P15S (S16 in human CR2) and T68Y (Y69 in human CR2) substitutions, allowed mouse CR2 to bind EBV with great efficiency. To allow a direct comparison of these sites to those with which the new anti-CR2 and other mAbs bind, the location on CR2 of these sites is also shown in Fig. 7, F and G.

#### Discussion

In these studies we have used a novel strategy to create a series of new and informative mAbs directed against human CR2 (Table I). Taking advantage of the complete lack of expression of endogenous CR2/CR1 in these *Cr2*<sup>-/-</sup> mice, along with the ability of strong adjuvants to in part bypass the immune deficiency in these mice, we were able to generate a robust immune response when immunizing mice with the SCR 1-2 fragment alone of human CR2. Importantly, a comparison with two previously described inhibitory mAbs revealed that the use of this strategy has allowed the creation of mAbs that recognize novel epitopes and one mAb, 171, that directly recognizes the CR2-C3d interface.

Of the four new anti-CR2 mAbs generated, three inhibit CR2 interactions with C3 fragment and gp350/220 ligands. This was first shown by ELISA analysis using purified ligands in the form of C3dg and the gp350/220 amino acid 1–470 fragment. In addition, ligand-receptor interactions in a more physiologic state were also disrupted. These inhibitory effects were demonstrated in an erythrocyte rosette analysis when C3dg is fixed to sheep erythrocytes through the action of the classical pathway, when using whole EBV and primary B cells in a transformation assay, and when C3 fragment-bound HIV particles are prebound in vivo to B cells using endogenous complement recognition and activation mechanisms. Thus, we believe that these are inhibitory mAbs that will have substantial general utility in studying CR2 as they block physiologically relevant receptor-ligand interactions.

We have also used a strategy of identifying mAb epitopes by determining the ability of individual mAbs to recognize linear peptides derived from CR2. Although this technique is limited in its ability to detect conformational epitopes generated by nonlinear amino acid sequences and does not work with each mAb (e.g., OKB7), we believe it has been highly informative with regard to our three inhibitory mAbs. Specifically, the most inhibitory mAb, 171, is shown by this technique to recognize an epitope that contains two of the eight contiguous CR2 amino acids positioned within the CR2-C3d interface. Thus, the role of mAb 171 is most



**FIGURE 7.** Structure-guided CR2 epitope mapping. *A*, The ribbon diagram showing the interface between CR2 (yellow) and C3d (blue). The CR2 loop containing 87-TPYR-90 is located at the top part of the interface. The side chains of 87-TPYR-90 of CR2 are labeled. *B*, CR2 surface representation showing the interface (in blue) with C3d. *C*, CR2 surface representation showing the epitope position of mAb171, 1048, and 994. The deep blue color patch represents the location of mAb171 epitope 87-TPYR-90 (compare with the location of 87-TPYR-90 loop in *A*), and the red patch indicates the location of the epitope of mAbs 1048 and 994. Notice that the red patch is adjacent to, but on the opposite side of, the CR2-C3d interface surface. *D* and *E*, CR2 surface structure at two different rotation angles (120 degrees away from each other) showing the positions of the five possible epitopes of FE8. The patch 1 (in blue) correspond to 88-PYRHGD-93. Patches 2–5 are in red and correspond to the following peptides respectively: patch 2 to 64-EYFNKYSS-71, patch 3 to 16-YYSTPI-21, patch 4 to 106-NGNKS-111, and patch 5 to 112-WCQANN-117. *F* and *G*, The two regions (red) containing epitope locations of mAb OKB7. Patch 1 represents the position of the linker peptide 64-EYFNKYSS-71, and patch 2 is the location of peptide 8-PILNGRIS-16. The two green spots are the location of S16 (P15 in mCR2) and Y69 (T68 in mCR2).

likely to directly interfere with C3d binding to CR2 by physically blocking it. This is evidence that the x-ray crystal structure of the complex exists in solution. In contrast, the next two inhibitory mAbs in rank order, 1048 and 994, recognize amino acid sequences that are adjacent to, but do not include, the CR2-C3d interface. Thus, they are most likely to interfere with C3d binding through steric hindrance presented by other parts of the Ab (the Fc or the second Fab). This is also consistent with our crystal structure.

In addition, we have been able to compare in some experiments the inhibitory capability of our new mAbs to previously described mAbs OKB7 and FE8. Although limited mAb OKB7 reagent availability did not allow a full range of experiments, it is clear that mAbs 171 and 1048, like FE8 (37), are substantially better inhibitors of CR2-C3 interactions than mAb OKB7, as assessed by rosette analysis. In addition, mAb 171 demonstrated a more robust ability to remove C3-bound HIV immune complexes from B cells than mAb FE8, although the limited number of analyses performed to date does not allow a firm conclusion to be reached regarding relative inhibitory capabilities of these two mAbs in a broader range of ligand-receptor interactions. The mechanism of inhibitory effects of mAb FE8 is less apparent, as this mAb was reported to have multiple peptide reactivities that do not fit a contiguous epitope in the crystal structure of CR2. The mechanism of inhibitory effects of mAb OKB7 on ligand binding is also not apparent from our studies, although the epitope was defined by a different method. It is likely to be steric in nature.

Interpretation of the results of studies presented herein has been greatly aided by the determination of the three-dimensional structure of human CR2 (40). This has allowed us to create a structure-guided epitope map, replacing those previously presented that were based on modeling techniques alone (37, 39, 46). Of interest, previous theoretical models are different with regard to several important features of CR2. These include the role of SCR 1 in ligand binding as well as the relative orientation of SCR 1 to SCR 2. Specifically, SCR 1 does not make major contacts with C3d in the crystal structure, while previous models created by ourselves and others had proposed this as an important protein-protein interaction site. The crystal structure suggests that SCR 1 helps to stabilize the SCR 2 binding site, or alternatively SCR 1-SCR 1 dimerization allows CR2 receptors to more effectively interact with polymeric ligand on the cell surface. The uniquely long interdomain linker of eight amino acids is seen to be important, not only in primarily orienting SCR 1 to SCR 2 in rotation as previously thought, but also in allowing SCR 1 and SCR 2 to pack against each other using hydrophobic interactions.

Given the identification of the residues LNGRIS in SCR 1 as a potential C3d contact region both by peptide work and by the mAb OKB7 (39, 46), the possibility cannot be excluded that SCR 1 may be able to separate from SCR 2 and reorient itself around the C3d structure to bind to it, even though the tight packing of the SCR 1–2 domains in the complex with C3d would argue against such an interdomain rotation. Thus, these and other findings with regard to the crystal structure of the CR2-C3d complex have forced us to re-examine not only the results of previous studies but also the methodologic assumptions, such as the value of site-specific mutagenesis without a known structure or without more direct studies of receptor-ligand interactions using nuclear magnetic resonance-based methods, that have guided previous work in this area. With regard to the latter point, although we believe that the use of peptides as targets for mAbs in epitope mapping is informative, and the location of the peptides on the three-dimensional structure of CR2 with this technique is very consistent with the relative ability

of the mAbs to block ligand binding, it is clear that only the determination of a cocrystal structure of CR2 in complex with Fab of these mAbs will allow us to fully understand the molecular nature of the inhibitory interactions. Likewise, until the three-dimensional structure of the CR2-gp350/220 complex becomes available, we will not be sure of the nature of this particular receptor-ligand interaction and its exact relationship to the CR2-C3d interaction.

Finally, although we have focused attention on the relationship between mAbs and the CR2-C3d contact site, two other potential target sites on the CR2-C3d for inhibitors were revealed in the crystal structure (40). The first is the hydrophobic interface between SCR 1 and SCR 2, the stability of which may be critical to ligand binding. This may be accessible when CR2 SCR 1-2 is extended in solution in the nonliganded state (41). It is possible, for example, that mAb FE8 is inhibitory because it interferes with the interdomain linker, which is the site of its primary peptide reactivity. This is seen to become tightly packed in the connection between SCR 1 and SCR 2 when bound to C3d in its crystal structure. Alternately this mAb may sterically interfere with SCR 1-SCR 2 packing by recognition of site 4 that lies in the V-shaped region between the SCRs (see Fig. 7, *D* and *E*). The other potential site for inhibitors is the SCR 1-SCR 1 dimer interface, which if present and functionally important in CR2 complexes on cell membranes, would present another unanticipated target for inhibition of receptor activities. Future studies will address these possibilities.

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