

## STUDIES OF THE EPSTEIN BARR VIRUS RECEPTOR FOUND ON RAJI CELLS

### II. A Comparison of Lymphocyte Binding Sites for Epstein Barr Virus and C3d<sup>1</sup>

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A comparison was made between the binding sites of two receptors that are believed to be closely associated on human B lymphocytes: complement receptor type two (CR<sub>2</sub>) that is specific for C3d fragments, and the receptor (EBVR) for Epstein Barr virus (EBV). Isolated fluid-phase CR<sub>2</sub> bound to C3d on erythrocytes (EC3d) and inhibited both B cell-EC3d rosettes and the agglutination of EC3d by anti-C3d, it failed to inhibit either the binding or superinfection of B cells by EBV. By contrast, isolated fluid-phase EBVR inhibited EBV B cell binding activity and superinfection but had no CR<sub>2</sub> activity. In addition, radiolabeled CR<sub>2</sub> bound to EC3d and anti-CR<sub>2</sub>-Sepharose, whereas radiolabeled EBVR did not. Purified fluid-phase C3d fragments inhibited EC3d rosette formation with CR<sub>2</sub><sup>+</sup>/EBVR<sup>+</sup> cells but did not inhibit EBV binding. However, EBV binding to B cells did inhibit EC3d rosette formation. Clones of human/mouse somatic cell hybrids made from CR<sub>2</sub><sup>+</sup>/EBVR<sup>+</sup> human B lymphoblastoid cell and CR<sub>2</sub><sup>-</sup>/EBVR<sup>-</sup> mouse myeloma cell parents expressed either EBVR or CR<sub>2</sub> but only rarely expressed both EBVR and CR<sub>2</sub>. This suggested that the genes for EBVR and CR<sub>2</sub> were located on two different human chromosomes. Thus it was concluded that CR<sub>2</sub> is probably not the binding site for EBV.

Human B lymphocytes bear a membrane binding site that is specific for the Epstein Barr virus (EBV). This EBV receptor (EBVR)<sup>5</sup> is essentially exclusive to B cells and is at least in part responsible for the B cell tropism of the virus (1-3). The exact biochemical nature and the normal cellular function of the receptor are not known, but several lines of evidence have

suggested identity or close association with the complement (C) receptor type two (CR<sub>2</sub>) that is specific for the d region of bound iC3b or C3d fragments (4). Expression of EBVR and CR<sub>2</sub> on B lymphocytes and the majority of B cell lines is coincidental (5-7), and induction of the two different receptor activities by theophylline is simultaneous (8). EBV binding is inhibited by pretreatment of cells with C3 and anti-C3 antibody, and EBV binding blocks formation of rosettes with erythrocytes coated with antibody and C3d (9).

The preceding paper (10) described extraction of EBVR activity from the EBVR<sup>+</sup> Raji cell. In this paper we compare the properties of this receptor material with those of purified CR<sub>2</sub>. We present evidence that indicates that EBVR and CR<sub>2</sub> are not the same.

#### MATERIALS AND METHODS

**Cells.** Growth and derivation of lymphoblastoid cell lines and somatic cell hybrids are described elsewhere (10); the only additional lymphoblastoid cell line used was Daudi (11).

**Somatic cell hybrids.** Human/mouse somatic cell hybrids were obtained from fusion of human lymphoblastoid cells and cultured mouse myeloma cells as described (10).

**Virus production and assay.** EBV was obtained from the spent culture media of P3HRI cells (10). Virus was assayed by superinfection of Raji cells followed by analysis of polypeptide synthesis in radiolabeled cells by using polyacrylamide gel electrophoresis on 7.5% slab gels (PAGE) and autoradiography (10). Virus was also assayed for binding to EBVR<sup>+</sup> cells by immunofluorescence with human antiviral antibody and fluoresceinated staphylococcal protein A (FITC-protein A) or F(ab')<sub>2</sub> anti-human IgG (10).

**Extraction of Raji cells and assay of EBVR activity in extracts.** A Raji cell extract containing EBVR activity (RE) was prepared by incubating cells at 4°C in 0.45 mM Tris HCl, pH 6.0, containing 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein inactivator units of Trasylol/ml (10). The EBVR activity in the material was assayed by measuring residual free virus in RE/virus mixtures that had been incubated together for 1 hr at 37°C (10).

**C components and C-coated sheep erythrocytes (E).** C components and nephritic factor were isolated from human plasma and used to prepare C receptor type 1- (CR<sub>1</sub>) reactive E coated with C3b fragments (EC3b) as described (12). EC3b was converted into CR<sub>2</sub>-reactive EC3d with KSCN-inactivated serum and plasmin (12). C3d fragments were isolated from trypsinized C3b bound to activated thiol-Sepharose (Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, NJ) as described (13).

**Preparation of CR<sub>2</sub>.** A 72,000-dalton glycoprotein representing membrane-shed CR<sub>2</sub> was isolated from 15 liters of Raji cell culture supernatant fluids as described (4). Briefly, an equal volume of saturated ammonium sulfate was added to spent culture medium, and the precipitate was centrifuged at 15,000 × G for 30 min. After dialysis against 20 mM sodium phosphate buffer, pH 7.5, the dissolved precipitate was chromatographed sequentially on DEAE-Sephacel, Sephadex G-150, and C3d-Sepharose. The isolated CR<sub>2</sub> was monovalent for C3d, because it inhibited anti-C3d agglutination and EC3d (see below). Purified CR<sub>2</sub> was trace-labeled with <sup>125</sup>I by the lactoperoxidase method (14) to a specific activity of 4.1 × 10<sup>4</sup> cpm/μg.

**Assay of CR<sub>2</sub>.** CR<sub>2</sub> activity was measured in terms of inhibition of agglutination of EC3d by anti-C3d (4). Isolated CR<sub>2</sub> also inhibited EC3d rosette formation with B cells, but assay for this activity was less sensitive than the anti-C3d inhibition assay (4). Serial 25-μl dilutions of test material were mixed with 25 μl of EC3d (1 × 10<sup>6</sup>/ml) and incubated on a shaker at

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<sup>5</sup> Abbreviations used in this paper: CR<sub>1</sub>, complement receptor type one, the C3b receptor; CR<sub>2</sub>, complement receptor type two, the C3d receptor; EBVR, Epstein Barr virus receptor; E, sheep erythrocyte; EC3b, sheep erythrocyte coated with C3b fragments; EC3d, sheep erythrocyte coated with C3d fragments; FITC, fluorescein isothiocyanate; RE, receptor extract.

37°C for 15 min, after which time 25  $\mu$ l of a 1/24 dilution of anti-C3d serum (kindly provided by Brian Tack, Scripps Clinic and Research Foundation, La Jolla, CA) were added. After another 15 min shaking at 37°C, the cells were allowed to settle for development of agglutination patterns.

**Preparation of antibody to CR<sub>2</sub>.** Antibody to CR<sub>2</sub> was prepared by weekly immunization of rabbits with 100  $\mu$ g purified CR<sub>2</sub> emulsified in Freund's complete adjuvant. Immune serum obtained after 6 wk completely inhibited binding of EC3d to CR<sub>2</sub>-bearing B cells and had no effect on the binding of EC3b to CR<sub>1</sub>-bearing B cells. F(ab')<sub>2</sub> fragments immunoprecipitated a single 72,000-dalton glycoprotein from <sup>125</sup>I-labeled B cell lines (4).

**Preparation of anti-CR<sub>2</sub>-Sepharose and bovine serum albumin- (BSA) Sepharose.** F(ab')<sub>2</sub> anti-CR<sub>2</sub> antibodies, prepared with pepsin and Sephadex G-150 chromatography (15), and BSA (Miles Laboratories, Elkhart, IN) were conjugated to Sepharose CL-4B (Pharmacia, Piscataway, NJ) with cyanogen bromide (16) at a ratio of 10 mg protein/ml gel.

## RESULTS

**Comparison of EBVR and CR<sub>2</sub> expression on extracted Raji cells.** EBVR<sup>+</sup>, CR<sub>2</sub><sup>+</sup> Raji cells were extracted under conditions that were previously shown to remove EBVR activity (10). After extraction, untreated and treated cells were resuspended in media containing 10  $\mu$ g/ml cyclohexamide and were compared for expression of EBVR and CR<sub>2</sub> (Table I). The percentage of cells that bound virus as determined by immunofluorescence decreased from about 86% to 25%. The intensity of virus-specific fluorescence on extracted cells that still bound virus was also reduced. This change in virus-binding ability contrasted sharply with the undiminished ability of the extracted cells to form EC3d rosettes. This result provided the first indication of differences between the EBVR and CR<sub>2</sub> membrane components.

**Comparison of RE and CR<sub>2</sub>. Inhibition of superinfection.** The ability of partially purified monomeric CR<sub>2</sub> (obtained at the G-150 step in the purification) to bind virus was compared with that of the RE by using the superinfection assay. Previous work had shown that preabsorption of virus with RE inhibits the ability of the virus to bind to and superinfect fresh Raji cells (10). P3HR1 virus was preabsorbed for 1 hr at 37°C with 500  $\mu$ l of CR<sub>2</sub> or with 500  $\mu$ l of RE. The absorbed virus was then used to superinfect fresh Raji cells. Six hours after infection, <sup>35</sup>S-methionine (10  $\mu$ Ci/ml) was added, and after 24 hr the cells were harvested, disrupted, and analyzed by PAGE and autoradiography (Fig. 1). The four polypeptides that are prime indicators of successful superinfection in this system (17) were present in the cell infected with virus preabsorbed with CR<sub>2</sub>, but were reduced or absent in those infected with virus preabsorbed with RE.

The same preparations were then compared in an assay to detect CR<sub>2</sub> activity. CR<sub>2</sub> material inhibited agglutination of EC3d by anti-C3d to a dilution of 1/3000; by contrast, undiluted and serial dilution of RE failed to inhibit EC3d agglutination by anti-C3d. These reciprocal assays provided further evidence that the same molecule was not responsible for both C3d- and EBV-binding activities.

**Binding to EC3d.** The ability of fluid-phase EBVR and CR<sub>2</sub> to bind to EC3d was further assessed by using a direct binding method. <sup>125</sup>I-labeled RE and partially purified <sup>125</sup>I-labeled CR<sub>2</sub> were incubated for 30 min at 37°C with EC3d, EC3b, or E. These red cells were washed, and the radioactivity that re-



**Figure 1.** PAGE electrophoresis of Raji cells superinfected with P3HR1 virus that has been preabsorbed with RE or CR<sub>2</sub> or an extraction buffer control. Superinfected cells were labeled with <sup>35</sup>S-methionine 6 hr after infection, harvested and disrupted after 24 hr in buffer containing sodium dodecyl sulfate, and analyzed by PAGE and autoradiography. (A) preabsorbed with extraction buffer control; (B) preabsorbed with RE; (C) preabsorbed with CR<sub>2</sub>. Numbers on the right indicate four virus-induced polypeptides that are prime indicators of successful superinfection.

mained bound was determined (Table II). EC3d bound 10% of the CR<sub>2</sub> counts added but fewer than 1% of the RE counts. Fewer than 1% of both CR<sub>2</sub> and RE counts bound to E and EC3b even when the radioactivity added was increased more than 10-fold.

**Binding to anti-CR<sub>2</sub>-Sepharose.** In order to determine whether RE contained any material which would react with anti-CR<sub>2</sub> antibody, <sup>125</sup>I-labeled RE and CR<sub>2</sub> were absorbed through columns of anti-CR<sub>2</sub>-Sepharose. Parallel absorption through BSA-Sepharose columns was included as a control for non-specific binding. There was no difference in the amount of <sup>125</sup>I-labeled RE that bound to the anti-CR<sub>2</sub>-Sepharose vs the BSA-Sepharose. By contrast, about 75% of the applied <sup>125</sup>I-labeled CR<sub>2</sub> bound to the anti-CR<sub>2</sub>-Sepharose, whereas much less bound to BSA-Sepharose (Table III).

**EBV binding to C3d-treated Daudi cells.** Daudi cells like Raji cells express both EBV and C3d receptors. However, unlike Raji cells, the number of CR<sub>2</sub> on Daudi cells is low enough for rosette formation by EC3d to be inhibitable by preincubation of the cells with relatively low concentrations of purified C3d fragments (31,000 daltons). Daudi cells were therefore used to compare the effect of C3d fragment preincubation of the cells on EC3d rosette formation and EBV binding. Aliquots of 5  $\times$

**TABLE I**  
Effects of a 3-hr extraction on Raji cells

Treatment	Percent of Cells Binding		Percent of Cells Viable
	EBV <sup>a</sup>	EC3d	
RPMI	86	100	96
Extraction buffer	25	100	93

<sup>a</sup> EBV binding was visualized by human antiviral antibody and FITC-protein A.

TABLE II  
Binding of <sup>125</sup>I-labeled RE and CR<sub>2</sub> to C-coated E

Addition	Counts per Minute Bound		
	EC3d	EC3b	E
CR <sub>2</sub> 60,000 cpm	5676	210	210
RE 60,000 cpm	276	255	267
RE 750,000 cpm	1000	970	900

TABLE III  
Column chromatography of <sup>125</sup>I-labeled RE and CR<sub>2</sub> on anti-CR<sub>2</sub>-Sepharose

Addition	Counts per Minute Bound	
	Anti-CR <sub>2</sub> -Sepharose	BSA-Sepharose
RE, 10 <sup>6</sup> cpm	25,415	26,673
CR <sub>2</sub> , 10 <sup>6</sup> cpm	750,125	35,125

TABLE IV  
Effect of purified C3d fragments on the ability to bind either EBV or EC3d

Pretreatment of Cells	Percent Binding <sup>a</sup>	
	EC3d	EBV <sup>b</sup>
PBS + paraformaldehyde	78	81
C3d + paraformaldehyde	4	87

<sup>a</sup> Cells binding four or more EC3d per cell were considered positive.

<sup>b</sup> EBV binding was visualized by human antiviral antibody and FITC-protein A.

TABLE V  
Expression of EBVR and C receptors on human-mouse somatic cell hybrid cells<sup>a</sup>

Hybrid	Clone	EBV-R <sup>b</sup>	CR <sub>1</sub> <sup>c</sup>	CR <sub>2</sub> <sup>d</sup>
RPC/GM2080	cl. 2	24	2	0
RPC/GM1247	cl. 3	5	0	7
SP2/CaCu	cl. 6	20	0	0
SP2/Cord	cl. 1	0	4	4
	cl. 63	0	12	0
	cl. 76	10	12	0
	cl. 94	42	0	0
	cl. 95	26	0	0

<sup>a</sup> Results are expressed as the percent of cells bearing receptors.

<sup>b</sup> EBVR expression was determined by immunofluorescence by using virus, F(ab')<sub>2</sub> antiviral antibody, and FITC-labeled F(ab')<sub>2</sub> anti-human IgG.

<sup>c</sup> CR<sub>1</sub> was assayed by rosette formation with EC3b.

<sup>d</sup> CR<sub>2</sub> was assayed by rosette formation with EC3d.

10<sup>5</sup> Daudi cells were incubated with 80 μl (40 μg) C3d or with phosphate-buffered saline (PBS) for 15 min on ice. Four milliliters of 0.1% paraformaldehyde were added, and the cells were pelleted, washed twice in RPMI, and assayed for EBV binding and EC3d binding as usual. The C3d fragments inhibited EC3d rosette formation by more than 90%, but failed to reduce EBV binding by any significant amount (Table IV).

**Expression of CR<sub>2</sub>, CR<sub>1</sub>, and EBVR on hybrid cells.** Somatic cell hybrids were prepared by fusing mouse myeloma cells with human lymphoblastoid cells that expressed EBVR, CR<sub>2</sub>, and CR<sub>1</sub> (the C receptor for C3b). Hybrid clones were examined for expression of CR<sub>2</sub> by EC3d rosette formation, CR<sub>1</sub> by EC3b rosette formation, and EBVR by immunofluorescence staining with virus and F(ab')<sub>2</sub> antibody fragments. Only one hybrid expressed both EBVR and CR<sub>2</sub> in detectable amounts, one expressed CR<sub>2</sub> but not EBVR, 15 expressed only EBVR, one expressed both CR<sub>2</sub> and CR<sub>1</sub>, eight expressed CR<sub>1</sub> alone, and three expressed EBVR and CR<sub>1</sub>; none expressed all three receptors. Expression on eight of these hybrid clones is tabulated in Table V.

#### DISCUSSION

The EBVR is of interest both as major determinant of the species and cell restriction of EBV and as a molecule that is normally coexpressed with CR<sub>2</sub>. Previous workers have reported that the receptors for EBV and C3d represent the same

or very closely associated structures. Lymphoblastoid cell lines usually express both EBVR and CR<sub>2</sub> (5–8); complexes of EBV or C3d and antibody reciprocally block the receptors (9), and membrane stripping of one receptor coincidentally removes the other, whereas stripping of Fc receptors, IgM, or β<sub>2</sub>-microglobulin has no such effect (18).

More recent work, however, has cast doubt on the association of EBVR and CR<sub>2</sub>. Although the majority of cell lines undoubtedly do coexpress EBVR and CR<sub>2</sub>, and the receptors may be coincidentally reexpressed (6) or induced (8), there are reports of two Burkitt/epithelial cell hybrids that express EBVR but not CR<sub>2</sub> (19). Also, lymphocytes from patients with common variable agammaglobulinemia express CR<sub>2</sub> but not EBVR (20), and a number of null cell lines bind EBV but fail to bind C3d (21). Our recent success in extracting EBVR activity from Raji cells enabled us to make the direct comparison between fluid-phase CR<sub>2</sub> and EBVR that is reported in this paper, and to determine that EBV and C3d probably do not bind to identical sites on the same membrane component. The evidence in favor of this conclusion is manifold.

First, the loss of EBV binding to extracted Raji cells without apparent loss of C3d binding suggests separateness of the active EBVR and CR<sub>2</sub> sites. This suggestion is supported by the failure of RE to demonstrate any CR<sub>2</sub> activity despite its ability to behave as a fluid-phase EBVR, and is strengthened by the complementary failure of fluid-phase CR<sub>2</sub> to compete with EBVR<sup>+</sup> cells for EBV binding. It is further supported by the failure of radiolabeled RE to show any significant or preferential binding to EC3d, EC3b, or E alone under conditions that specifically bind 10% of the CR<sub>2</sub> counts added to EC3d. The specific activity of the radiolabeled purified CR<sub>2</sub> is clearly greater than that of the RE preparation, which contains more than 60 labeled polypeptides (10). However, increasing the amount of RE in the assay by as much as 10-fold still failed to evince any specific binding to EC3d. Also, in addition to this functional assay, which required preservation of biologically active molecules, we found that immobilized anti-CR<sub>2</sub> antibody was capable of specifically absorbing radiolabeled CR<sub>2</sub> but incapable of specifically absorbing radioactive RE.

To complement these experiments with fluid-phase reactants, we were able to confirm, by use of low m.w. C3d fragments, that blocking of cellular CR<sub>2</sub> can be achieved without interference with the EBVR site (9). Blocking of EC3d rosette formation by EBV, although not reported by others (9), has been achieved in our hands (data not shown). However, EBV is much larger than the C3d fragment and is thus presumably capable of steric inhibition of the C3d binding site.

A third line of evidence that EBVR and CR<sub>2</sub> are distinct is the observation that expression of EBVR and CR<sub>2</sub> on mouse/human somatic cell hybrids is rarely coincidental. Only one out of 29 hybrids examined expressed both receptors. Because it is known that mouse/human hybrids selectively lose their human chromosomes, these data suggest that CR<sub>2</sub> and EBVR are probably encoded by genes present on two different human chromosomes.

Our data and those of others then clearly indicate that EBVR and CR<sub>2</sub> are extremely unlikely to share active sites. However, we are still unable to answer the original two intriguing questions, Why are these two receptors usually coexpressed? and, When this happens, why are they always closely associated?

Magrath and co-workers (8) have speculated that the binding sites for EBV and C3d might be on different portions of the same molecule. Our failure to detect EBVR in concentrated spent Raji culture media might then reflect the known instability of the active EBVR site (10) and its loss of biologic activity as

detectable amounts of membrane-shed CR<sub>2</sub> accumulate. The failure to detect active CR<sub>2</sub> in RE is not likely to reflect inability of the procedure to extract enough CR<sub>2</sub> in 3 hr to be detectable by current assays. Even if there are extreme differences in the sensitivity of EBVR and CR<sub>2</sub> binding assays under optimal conditions, the anti-CR<sub>2</sub>-Sepharose might have been expected to preferentially absorb some RE. However, it is perhaps possible that the extraction procedure results in the proteolytic release of a fragment that contains only the EBVR binding site. The idea of a single molecule with two receptor sites is attractive. It is also in part supported by preliminary evidence (unpublished data) that anti-CR<sub>2</sub> antibody not only partially blocks EBV binding to Raji cells (presumably by steric inhibition), but also blocks EBV binding to functionally CR<sub>2</sub><sup>-</sup>/EBVR<sup>+</sup> human/somatic cell hybrids. This latter finding implies that the hybrid cells may in fact carry an inactive CR<sub>2</sub> molecule.

The probability of the receptor sites being covalently linked is nevertheless remote for other reasons. The anti-CR<sub>2</sub> antibody immunoprecipitates a 72,000-dalton glycoprotein both from the purified fluid-phase CR<sub>2</sub> preparation and from lymphocyte membranes solubilized with Nonidet P-40 (4); anti-RE immunoprecipitated a polypeptide with an apparent calculated m.w. of approximately 150,000, but not one of 72,000 (10). None of these experiments was performed under conditions that might be expected to break covalent bonds. Furthermore, we have recently obtained data with a panel of human/mouse somatic cell hybrids that assign EBVR to chromosome 7 (unpublished data). The only tentative assignment of CR<sub>2</sub> has been to chromosome 6 (22), a fact that, if true, greatly reduces any likelihood that the two receptors might be synthesized as one molecule.

A more likely possibility is that CR<sub>2</sub> and EBVR are two distinct molecules, perhaps bound by polar or ionic bonds, which have stabilizing or conformational effects on each other when in close proximity in the lymphocyte membrane. Isolation, purification, and insertion of CR<sub>2</sub> and EBVR into artificial membranes are approaches to answering these questions. The possibility that a ubiquitous human virus has adapted to using a cell surface molecule that has a close association with a functional immune receptor is an interesting one.

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