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Immunology

Epstein–Barr virus receptor of human B lymphocytes is the C3d receptor CR2

(complement/Epstein–Barr virus/monoclonal antibody)

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ABSTRACT **Identity of the Epstein–Barr virus (EBV) receptor with the complement receptor type 2 (CR2) was established in three sets of experiments using the monoclonal antibodies, HB-5 and anti-B2, which recognize a M_r 145,000 B-lymphocyte membrane protein that is CR2. First, the rank order for binding of fluoresceinated EBV to four lymphoblastoid cell lines (SB, JY, Raji, and Molt-4) was identical to the rank order for binding of HB-5 and anti-B2 by analytical flow cytometry. Second, pretreatment of cells with HB-5 followed by treatment with goat F(ab')$_2$ fragments to mouse IgG blocked binding of fluoresceinated EBV on SB, a B-lymphoblastoid cell line. Virus attachment was not inhibited by HB-5 alone, second antibody alone, rabbit anti-C3b receptor, or UPC10 (an irrelevant monoclonal antibody). Third, transfer of CR2 from SB to protein A-bearing *Staphylococcus aureus* particles, to which HB-5 had been absorbed, conferred on them the specific ability to bind ^{125}I-labeled EBV. We conclude that CR2 is the EBV receptor of human B lymphocytes.**

The possibility that the human B-cell surface receptor for Epstein–Barr virus (EBVR), a human herpesvirus associated with infectious mononucleosis, Burkitt lymphoma, and nasopharyngeal carcinoma, is related to receptors for the third component of complement (C3) has been recognized for several years (1, 2). The EBVR and C3 receptors were coincidentally expressed (1–3) and induced (4) on B-cell lines and on peripheral blood B lymphocytes. They cocapped (5) and were mutually depleted after membrane stripping (6). Uptake of virus by lymphoblastoid cells interfered with the binding of sheep erythrocytes coated with C3 fragments (1, 7) and sequential treatment with human C3, rabbit anti-C3 antibody, and goat anti-rabbit Ig blocked adherence of EBV (1). Subsequently, comparison of the ability of cells to bind ^{125}I-labeled EBV with their formation of rosettes with C3b and C3d-coated erythrocytes revealed a correlation between the receptors for EBV and C3d rather than C3b (8).

Three different types of cellular receptors for the major cleavage fragments of C3 have now been defined. The C3b receptor, or complement receptor type 1 (CR1), has primary specificity for C3b, the major cleavage fragment of C3, but it may also bind iC3b (9, 10), the first product of factor I cleavage, and C4b (11), the major cleavage fragment of the fourth component of complement. It is expressed on erythrocytes, phagocytes, B lymphocytes, some T lymphocytes, and glomerular podocytes (12–15) and has been shown to be a membrane glycoprotein with two allotypic forms of M_r 250,000 and M_r 260,000 (16, 17). The iC3b receptor, or complement receptor type 3 (CR3), has primary specificity for this cleavage fragment but may, in addition, bind C3d,g, the second product of factor I cleavage. This receptor is expressed on phagocytes and large granular lymphocytes having natural killer and antibody-dependent cytotoxic activities, but it is not expressed on B lymphocytes (18–21). It consists of two polypeptide chains of M_r 155,000–185,000 and M_r 95,000–105,000 (20, 22, 23). The C3d receptor or CR2 binds the C3d region of C3d,g, iC3b and, with less affinity, C3b. It is found on mature B lymphocytes and on certain B-cell lines (24–29). It has been characterized as a M_r 140,000–145,000 membrane protein (26, 27) that is recognized by the monoclonal antibodies (mAbs) termed anti-B2 (28) and HB-5 (29), respectively.

In the present study, these mAbs have been used to show that the EBVR and CR2 are quantitatively coexpressed on four cell lines, that binding of antibody to CR2 can prevent attachment of EBV, and that CR2 that has been immunoabsorbed onto particles of *Staphylococcus aureus* Cowan I strain (SACI) specifically binds EBV.

MATERIALS AND METHODS

Cell Lines. The hematopoietic cell lines B95-8 (30), SB (31), HSB-2 (31), JY (32), K562 (33), Molt-4 (34), and Raji (35) were grown in suspension culture at 37°C in 5% CO_2/95% air. They were diluted biweekly in 90% RPMI medium (GIBCO) and 10% fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Antibodies. HB-5 is an IgG2a mouse mAb (29) that has specificity for CR2 (27). The F(ab')$_2$ fragment was prepared by pepsin digestion of the purified immunoglobulin and was subsequently labeled to a specific activity of 2×10^6 cpm/μg with ^{125}I (36). Anti-B2 is an IgM mouse mAb obtained from Coulter that also has specificity for CR2 (26, 28). UPC10, an IgG2a mouse mAb with hapten specificity for β-2-6-linked fructosan, was obtained from Litton Bionetics. W6/32 is an IgG2a mouse mAb directed against a framework determinant of class I HLA molecules (37). LB3.1 is an IgG2a mouse mAb directed against a constant-region determinant of HLA-DR. Rabbit F(ab')$_2$ anti-CR1 (12) and rabbit IgG anti-C3d (38) were prepared as described. Unconjugated and fluorescein isothiocyanate (FITC)-conjugated goat F(ab')$_2$ anti-mouse F(ab')$_2$ [GAM F(ab')$_2$] and unconjugated and FITC-conjugated goat F(ab')$_2$ anti-rabbit F(ab')$_2$ (GAR) were obtained from Cappel Laboratories (Cochranville, PA). FITC-conjugated goat F(ab')$_2$ anti-mouse IgM (GAM IgM) was

Abbreviations: EBV, Epstein–Barr virus; EBVR, Epstein–Barr virus receptor; C3, third component of complement; CR1, complement receptor type 1, which has primary specificity for C3b; CR2, complement receptor type 2, which is expressed only by B lymphocytes and has primary specificity for the C3d region of C3d,g; SACI, *Staphylococcus aureus* Cowan I strain; FITC, fluorescein isothiocyanate; GAM F(ab')$_2$, goat F(ab')$_2$ anti-mouse F(ab')$_2$ fragment; GAM IgM, goat F(ab')$_2$ fragment anti-mouse IgM; GAR, goat F(ab')$_2$ anti-rabbit F(ab')$_2$ fragment; mAb, monoclonal antibody.

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Table 1. Relative median fluorescence intensity

Cell line	EBV	HB5	Anti-B2	Anti-CR1	W6/32*
JY	15	56	37	3†	2937
RAJI	12	35	31	0	703
SB	10	23	18	1†	2231
Molt-4	3	10	8	0	150
K562	0	2	2	0	1

Median fluorescence intensity was calculated by converting from arbitrary logarithmic units to a linear scale and subtracting median autofluorescence of the respective cell lines.

*Median fluorescence values produced by cellular uptake of different antibodies cannot be directly compared as the antibodies are of distinct immunoglobulin classes and subclasses and have variable affinities.

†These lines express low but detectable amounts of CR1.

determinant), on these five cell lines differed from that of FITC-conjugated EBV (Table 1). In two additional experiments comparing the uptake of FITC-conjugated EBV and HB-5, the rank order of fluorescence intensity of virus and mAb-stained cells changed to SB > Raji > JY >> Molt-4 >>> K562. Thus, when the relative rank order of the cell lines changed for binding of one ligand, it also changed in the same manner for the other two ligands. In all experiments, the fluorescence intensity of the T-cell line, Molt-4 was severalfold less than that of the B-lymphoblastoid lines. Quantitation of the number of CR2 molecules on these human cell lines, using the ^{125}I-labeled F(ab')$_2$ fragment of HB-5, confirmed the rank order of CR2 expression obtained by flow cytometry with the three B-cell lines expressing an average of 24,000–63,000 sites per cell, with Molt-4 expressing an average of 8000 sites per cell and HSB-2 being negative in an assay that would detect <200 sites per cell.

Inhibition of EBV Binding to SB Cells with mAbs Directed to CR2. Demonstration that the relative numbers of EBV and CR2 receptors were identical on five cell lines prompted analysis of the capacity of the HB-5 mAb to block adherence of FITC-conjugated EBV to B-lymphoblastoid cells. SB cells that had been incubated with UPC10 or anti-CR1 alone or with a second antibody alone bound FITC-conjugated EBV, as demonstrated by the greater fluorescence of those cells (Fig. 1 *B* and *C*) compared to autofluorescence of SB cells that had not been exposed to FITC-conjugated EBV (Fig. 1*A*). Preincubation of cells with a saturating concentration of HB-5 (40 μg/ml) and a second antibody abolished subsequent uptake of FITC-conjugated EBV, whereas treatment with only HB-5 had no inhibitory effect (Fig. 1*D*). Pretreatment of three other EBVR-positive cell lines, Raji, JY, and Molt-4 with HB-5 and a second antibody also abolished uptake of FITC-conjugated EBV, but similar treatment of the EBVR-negative cell line HSB-2 did not decrease the nonspecific binding of virus to these cells (data not shown). Treatment of cells with HB-5 plus anti-B2 but without second antibody did not inhibit uptake of FITC-conjugated EBV by SB, nor did the presence of rabbit IgG to C3d fragments during incubation with FITC-conjugated virus (data not shown). W6/32, which identifies class I antigens that are expressed 15- to 100-fold more densely than CR2 and LB3.1, which was analyzed because of a previous report that a nearly monospecific heteroserum to HLA-DR could block absorption of EBV (42), did not prevent adherence of FITC-conjugated EBV to SB in the presence or absence of second antibody (data not shown).

Specific Binding of ^{125}I-Labeled EBV to SACI Particles Bearing Immunoabsorbed CR2. To assess directly the capacity of CR2 to bind EBV, SACI particles bearing HB-5 and incubated with detergent lysates of SB cells under conditions that in other experiments led to the unique uptake of CR2 (Fig. 2) were assayed for their ability to bind ^{125}I-labeled EBV. Control SACI particles bearing HB-5 and exposed to detergent lysates of the CR2-negative HSB-2 cell line and particles bearing UPC10 and preincubated with detergent lysates of SB cells were also assayed for their ability to bind ^{125}I-labeled EBV. Particles to which CR2 had been immunoabsorbed bound 3-fold more ^{125}I-labeled EBV than did equivalent numbers of control particles bearing irrelevant mAbs (UPC10) and exposed to SB lysates, or bearing HB-5 and exposed to CR2-negative cell lysates at each of four inputs of the labeled virion preparation (Fig. 3*B*). The 3-fold increment in binding of ^{125}I-labeled EBV by the CR2-bearing

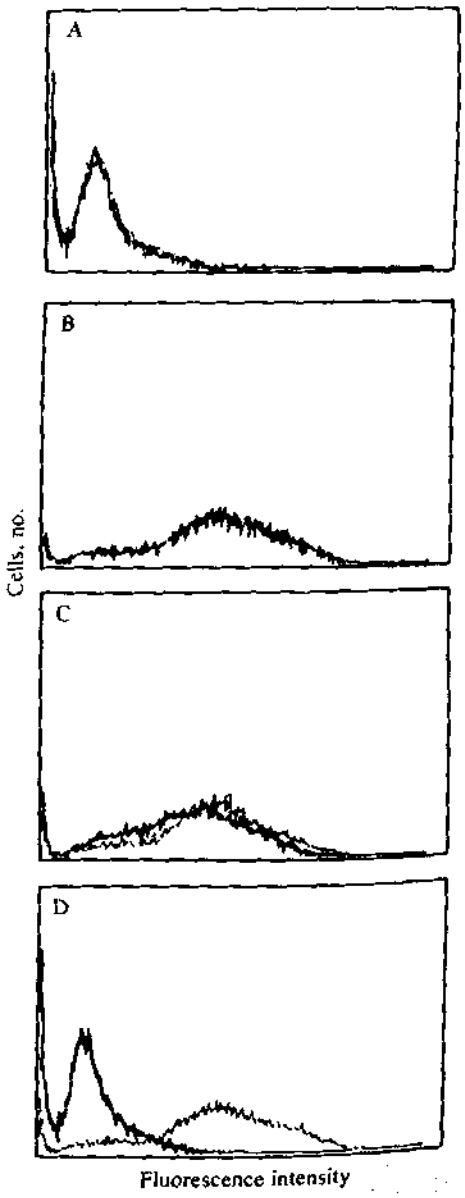


FIG. 1. Relative cell numbers are on a linear scale and fluorescence intensity is on a logarithmic scale. (*A*) Autofluorescence of cells preincubated either with anti-CR1 followed by unfluorescein-ed GAR (—) or with HB-5 followed by GAM F(ab')$_2$ (—). (*B*) Fluorescence of SB cells preincubated with UPC10 alone (—) or followed by GAM (Fab')$_2$ (—) and subsequently incubated with FITC-conjugated EBV. (*C*) Fluorescence of SB cells preincubated with anti-CR1 alone (—) or followed by GAR (—) and subsequently incubated with FITC-conjugated EBV. (*D*) Fluorescence of SB cells preincubated with HB-5 alone (—) or followed by GAM (Fab')$_2$ (—) and subsequently incubated with FITC-conjugated EBV.

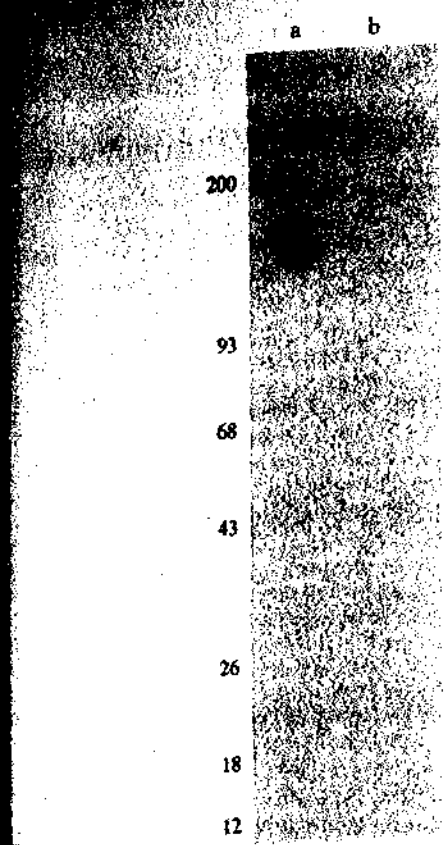


FIG. 2. Autoradiograph of $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled membrane proteins immunoprecipitated from SB cells by HB-5 (lane a) or UPC10 (lane b). Molecular weights are shown as $M_r \times 10^{-3}$.

SACI particles was similar to the relative uptake of this labeled EBV preparation by CR2-positive intact SB cells versus the receptor-negative HSB-2 cells (Fig. 3*A*). To assure that enhanced uptake of ^{125}I-labeled EBV by CR2-bearing SACI particles was specific for this membrane protein, particles bearing class I HLA antigens were prepared by sequential incubation with W6/32 and detergent lysates of SB or HSB-2 and assayed for ^{125}I-labeled EBV uptake. Binding of ^{125}I-labeled EBV to these particles was no different than to control particles (as in Fig. 3*B*).

DISCUSSION

Previous studies have strongly suggested that receptors for cleavage fragments of C3 on B lymphocytes and lymphoblastoid cell lines were spatially and perhaps structurally related to receptors for EBV (1–8). However, these perceptive experiments were limited by lack of knowledge concerning the membrane proteins mediating C3 receptor function, such that a precise definition of the relationship between the complement and viral receptors was not possible (43). The recent identification of mAbs specific for CR2 (26–29), combined with the earlier characterization of CR1 and development of a monospecific antibody (12), has permitted direct analysis of the role of these receptors in the uptake of EBV by B lymphocytes.

Three types of experiments using the anti-C3 receptor antibodies were performed: determination of whether a correlation existed between the relative numbers of EBVRs and either CR1 or CR2 on lymphoblastoid cells, examination of the capacity of the anti-receptor antibody HB-5 to specifically block EBV binding to lymphoblastoid cells, and analysis of the binding of EBV to purified immunoabsorbed CR2. The rank order of five cell lines for binding of FITC-conjugated EBV, as determined by cytofluorography, was the

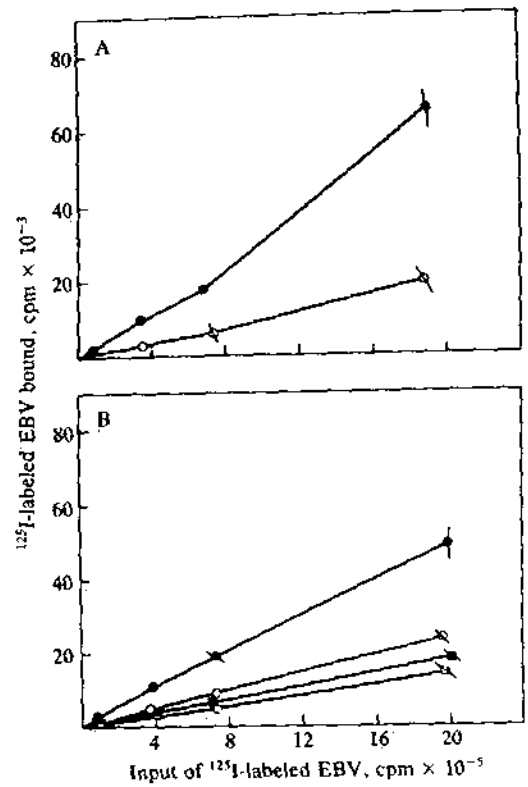


FIG. 3. (*A*) ^{125}I-labeled EBV binding to human cell lines SB (●) and HSB-2 (○). (*B*) ^{125}I-labeled EBV binding to SACI particles coated with monoclonal antibody and cell lysate; HB-5, SB lysate (●); UPC10, SB lysate (○); HB-5, HSB-2 lysate (■); UPC10, HSB-2 lysate (□).

same as that for binding of the two mAbs specific for CR2 but differed from that for uptake of anti-CR1 (Table 1). These results not only extended in a quantitative manner the correlation noted previously between cellular expression of the EBVR and C3d binding activity, but also identified the specific membrane protein with which uptake of EBV was correlated. The latter observation was important because of two recent findings, that CR2 could with lesser affinity bind C3b (27), and that another C3d binding receptor distinct from CR2 was present on neutrophils and monocytes (44, 45). Thus, the mAbs HB-5 and anti-B2 are more discriminating ligands than are fragments of C3. This specificity of the mAb was critical to the experiment, demonstrating inhibition by HB-5 and a second antibody of EBV binding to SB cells (Fig. 1). The inability of HB-5 alone to block virus uptake suggested that the mAb interacted with an epitope on the receptor that was distinct from the site occupied by the virus. The third type of experiment, demonstrating that the EBVR was identical with CR2, took advantage of this observation by using HB-5 coupled to SACI particles for absorption of CR2 (Fig. 2). SACI particles with CR2, but not those with HB-5 alone or with immunoabsorbed class I antigens, were as capable of binding EBV as intact donor SB cells (Fig. 3). This finding directly demonstrated that CR2 had the functional capability of an EBVR and, combined with the blocking studies, indicated that CR2 was the unique EBVR of B lymphocytes.

The previous finding that fluid phase C3 fragments can inhibit rosette formation by erythrocytes coated with C3d but cannot prevent absorption of EBV (1, 7) suggests that virus and C3d,g may bind to different epitopes on the same molecule, although very high affinity of EBV for the same recognition site may also explain this observation. Altered affinities for a common receptor may also provide an explanation for the finding that B lymphocytes from patients with

some forms of agammaglobulinemia can rosette with erythrocytes that have been coated with antibody and C3d, but do not absorb EBV (46). A previous study, which concluded that the EBVR and CR2 were nonidentical (7, 47), used a rabbit anti-CR2 identifying a M_r 72,000 protein that may have represented a proteolytic degradation product of the intact receptor (26, 27). Indeed, the EBV binding protein extracted from Raji cells in that study had a M_r of 150,000, which is now known to be similar to that of CR2 (26, 27).

Transformation by EBV has provided a major biological tool for maintaining B lymphocytes in culture. The factors governing susceptibility to infection and transformation by virus among diverse B-cell populations have not been well understood. Recently, the ontogeny of the B-cell surface antigen defined by anti-B2 and HB-5, and its identity with CR2 have been described (26–29, 48). Demonstration that this membrane protein also functions as the EBVR explains the tropism of EBV for specific B-lymphocyte populations and will permit exploration of additional factors that determine whether transformation or cell lysis ultimately occur.

The identification of a single membrane protein as the receptor for C3d and EBV is intriguing. EBV has long been recognized as a potent T-cell-independent polyclonal B-cell activator (49, 50). However, the precise mechanism of activation has remained obscure. Likewise an immunomodulatory role for the cleavage products of C3 has been postulated (51), but it has been difficult to clarify the nature of the C3 fragments and the C3 receptors involved. Recently, it has been suggested that antibody to CR1 may enhance maturation of B lymphocytes (52). It is likely that both EBV and C3d bind to CR2 as multimolecular complexes, the former as a biological membrane consisting of repetitive antigen units and the latter associated with immune complexes after cleavage of bound C3b by CR1 and I into C3d,g. One might speculate that the ability to cross-link CR2 constitutes an effector mechanism in the process of early B-cell activation. Recognition of a dual role for the M_r 145,000 protein identified by HB-5 as CR2 and the EBV receptor should facilitate further investigation.

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