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Purification of the Epstein-Barr Virus/C3d Complement Receptor of Human B Lymphocytes: Antigenic and Functional Properties of the Purified Protein

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The Epstein-Barr virus/C3d receptor (CR2) of human B lymphocytes was purified to homogeneity from Raji cells by immunoaffinity chromatography. The average yield of the 145-kilodalton receptor was 400 pmol (50 μg) per 10^{10} cells, representing an approximate 75% recovery. The isolated 145-kilodalton protein was antigenically and functionally intact as it reacted with several anti-CR2 monoclonal antibodies and bound purified Epstein-Barr virus and C3d,g. These findings with the purified molecule provide an unequivocal demonstration of the dual receptor functions of this protein.

Numerous studies during the last decade have indicated that the Epstein-Barr virus (EBV) receptor is closely associated on the B-cell surface with the C3d complement receptor (CR2) (5–7, 14, 18, 19). Recent experiments with monoclonal antibodies (MAbs) and polyclonal antibodies (3, 4, 13) have provided strong evidence that a single 145-kilodalton (kDa) B-cell membrane protein subserves both EBV and C3d receptor functions. We recently found that several MAbs directed against this 145-kDa protein induce B-cell mitogenesis and differentiation (12; B. S. Wilson, J. L. Platt, and N. E. Kay, Blood, in press), indicating that this molecule was linked to a pathway of B-lymphocyte activation. To permit further biochemical and functional studies of the EBV/C3d receptor, a rapid, high-yield purification procedure was developed. The method described here possesses certain advantages over another recently published method (9). This report also presents the first description of the antigenic and ligand-binding properties of the purified molecule.

EBV and the AD169 strain of cytomegalovirus (CMV) were isolated from phorbol ester-induced B95-8 cells (11) and foreskin fibroblasts (1) as previously described; Rauscher-Murine leukemia virus (R-MuLV) was kindly donated by John Elder (Scripps Clinic & Research Foundation, La Jolla, Calif.). OKB7 and AB-1 MAbs were generously provided by Ortho Diagnostics, Inc., Raritan, N.J., and by Barry Wilson, University of Michigan, Ann Arbor, Mich., respectively; HB-5 MAb was purified from the THB-5 hybridoma cell line obtained from the American Type Culture Collection, and anti-B1 was purchased from Coulter Immunology, Hialeah, Fla. The MAbs were purified as previously described (2). HB-5 was biotinylated as recommended by the manufacturer (Enzo Biochem, New York, N.Y.). Streptavidin-horseradish peroxidase was purchased from Amersham Corp., Arlington Heights, Ill. An immunomatrix column was prepared by cross-linking 20 mg of purified HB-5 MAb to 5 ml of protein A-agarose (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) with 20 mM dimethyl pimelimidate (Pierce Chemical Co., Rockford, Ill.) as previously described (15).

The 145-kDa protein was purified from the Raji B lymphoblastoid cell line by immunoaffinity chromatography. Raji cells (2×10^{10}) were washed twice in cold phosphate-buffered saline (PBS) (pH 7.4) and suspended in 400 ml of 10 mM phosphate buffer (pH 8.0) containing 3.3% Brij 96 (Sigma Chemical Co., St. Louis, Mo.), 3 mM sodium azide, 50 mM iodoacetamide, 5 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 0.2 trypsin inhibitory U/ml of Aprotinin (Sigma). After thorough mixing at 4°C for 30 min, the nuclei and debris were removed by high-speed centrifugation. Sodium deoxycholate (DOC) in 10 mM Tris hydrochloride (pH 8.0) was added to the supernatant to achieve a final concentration of 1%. After mixing for 60 min at 4°C, the supernatant was incubated with constant mixing for 18 h with 2 ml of packed fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring, La Jolla, Calif.). The mixture was then centrifuged, and the supernatant was applied to the HB-5 immunomatrix column. The immunomatrix was batch-washed four times with 200 ml of 10 mM Tris hydrochloride (pH 8.0) containing 0.5% DOC and four times with 200 ml of 10 mM Tris hydrochloride (pH 8.0) containing 150 mM NaCl and 0.5% DOC. The immunomatrix was next poured into a column and eluted with 7 to 10 ml of 50 mM diethylamine (pH 11.5) (Kodak, Rochester, N.Y.) containing 0.5% DOC. The pH of the CR2-containing eluate was immediately adjusted to 8.0 by addition of solid glycine. The protein was then rechromatographed on the immunomatrix column to remove minor contaminants. Purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8) under reducing conditions; the gels were silver stained by the method of Morrissey (10). The protein concentration of the receptor was estimated by comparing densitometric scans of the receptor with known amounts of standard protein markers (Bio-Rad Laboratories, Richmond, Calif.) run in adjacent lanes of the gels. C3d,g was purified by a slight modification of a published method (9).

An enzyme-linked immunosorbent assay (ELISA) was used to assess the antigenic integrity of purified CR2. Various amounts (0.25 to 4 ng) of the purified 145-kDa protein in 100-μl portions in 0.1 M bicarbonate buffer (pH 9.5) were permitted to bind to 96-well microtitration plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) for 18 h at 4°C. The wells were blocked with 0.5% nonfat dry milk in PBS (pH 7.4) containing 0.05% antifoam A (Sigma) (blotto buffer) for 1 h at 37°C. Various MAbs (1 μg of each) in 100 μl of blotto buffer were added to the wells and incubated for 1

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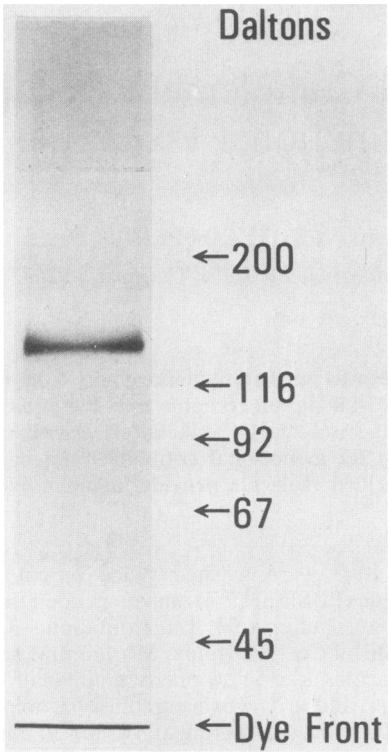

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the final stage of receptor isolation. A total of 30 ng was analyzed on a 7% gel under reducing conditions, after which the gel was silver stained. Daltons are given in thousands.

h at 22°C. After three washes with blotto buffer, 100 μl of a 1:500 dilution of biotinylated anti-mouse immunoglobulin (Vector Laboratories, Burlingame, Calif.) in blotto buffer was added, and the plates were incubated for 1 h at 22°C. The wells were again washed and then incubated for 30 min at 22°C with 100 μl of a 1:250 dilution of avidin-glucose oxidase (Vector). After further washes, the reaction was developed by the addition of chromogenic substrate (140 μg of 2,2′-azino-bis-3-ethylbenzthiazoline-sulfonate per ml, 7 μg of peroxidase per ml, 19 mg of glucose per ml in 0.1 M phosphate buffer [pH 6.0]). The reactions were read at A_{405} in a Titertek Multiskan MC ELISA plate reader (Flow Laboratories, Inc., McLean, Va.). Controls included wells precoated either with buffer alone or with bovine serum albumin (BSA). An irrelevant MAb, anti-B1, was also reacted with wells coated with the 145-kDa protein.

A dot blot immunoassay was employed to examine the ligand-binding properties of purified CR2. Various amounts (0.07 to 10 ng) of purified EBV, CMV, R-MuLV, C3d,g, or ovalbumin (0.01 to 1.75 μg) were blotted onto nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) by using a dot blotting apparatus (Bio-Rad). The virus samples were then blocked with blotto buffer containing 2.0% nonfat dry milk, whereas the C3d,g and ovalbumin samples were blocked with 2.0% BSA in PBS. After 30 min at 4°C, the blots were rinsed briefly in PBS and then incubated for 60 min at 22°C with 40 to 80 ng of purified receptor in 1.0 ml of blotto buffer containing 0.5% nonfat milk (virus blots) or in 1.0 ml of 0.1% BSA in PBS (C3d,g and ovalbumin blots); these two buffers also served as the reagent diluents and wash buffers for the virus and protein blots, respectively, in subsequent steps. The blots were rinsed three times and then incubated for 60 min at 22°C with 1 ml of a 1:500 dilution of biotinylated MAb HB-5 or, as a control, anti-B1, in wash buffer. The blots were again washed and then incubated for 30 min at 22°C with 1 ml of a 1:500 dilution of streptavidin-horseradish peroxidase. After three additional washes with the appropriate buffers, they were rinsed with PBS before the addition of substrate consisting of 2.5 ml of H_2O_2 and 2.5 ml of 4-chloro-1-naphthol (Kirkegaard and Perry Laboratories). After color development, the reaction was stopped by rinsing the blots with H_2O. Competition assays were carried out as described above, except that 40 ng of the 145-kDa protein was preincubated with 6 μg of either OKB7 or AB-1 anti-receptor MAbs for 60 min at 22°C before its addition to the ligand blots.

The protein isolated from the HB-5 immunomatrix column was free of contaminants and had a molecular size of 145 kDa (Fig. 1). Analyses of five different preparations indicated an average yield of 50 μg of the 145-kDa protein per 10^{10} Raji cells, as determined by comparison with protein standards included in the same gel runs and similarly scanned by densitometry. This method of quantitation was validated by amino acid analyses of the purified receptor (data not shown) and an estimated M_r of 120,000 (17).

ELISA analyses showed that the purified 145-kDa protein reacted with OKB7, HB-5, and AB-1 MAbs but not with anti-B1, an irrelevant MAb, in a dose-dependent manner (Fig. 2). As little as 0.25 to 0.5 ng of the protein was detected

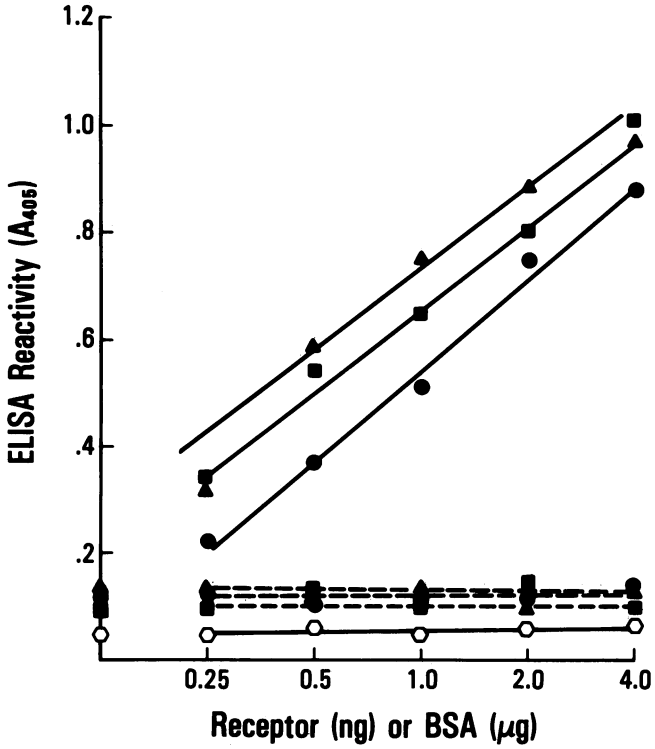

FIG. 2. ELISA of the purified receptor by using MAbs. Various amounts (0.25 to 4 ng) of the purified receptor (——) or BSA (-----) were coated onto plastic 96-well plates and analyzed for binding to AB-1 (▲), HB-5 (■), OKB7 (●), or anti-B1 (○) MAbs. Binding of the MAbs was detected by incubation with biotinylated anti-mouse immunoglobulin and avidin glucose oxidase. Values obtained for uncoated wells containing all other reactants are shown on the ordinate.

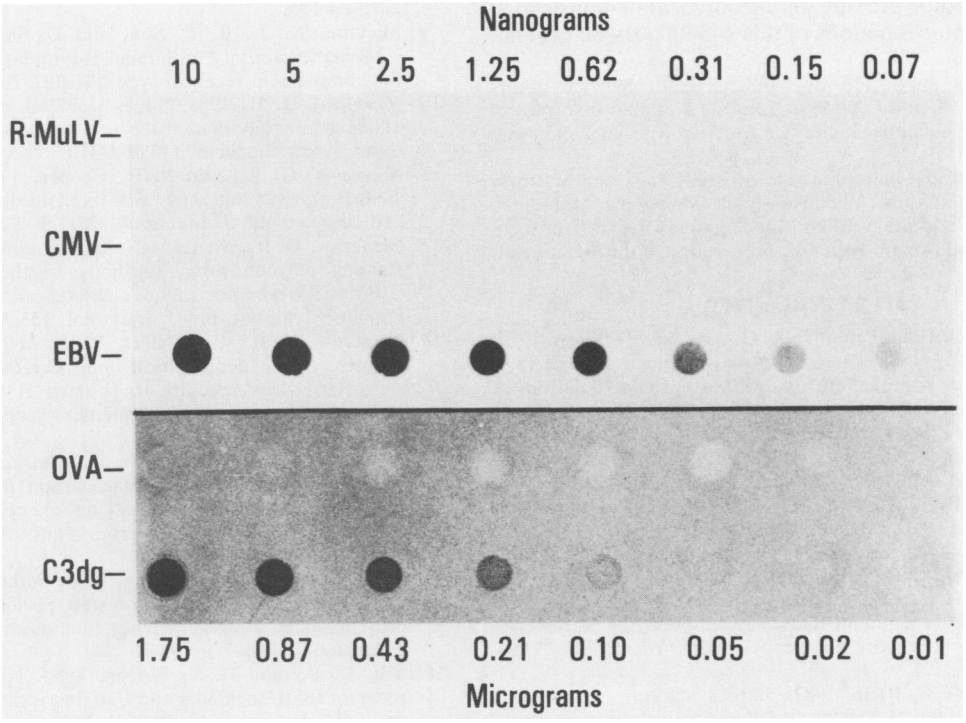

FIG. 3. Dot blot immunoassay for binding of the 145-kDa protein to EBV and C3d,g. A total of 40 ng of purified CR2 was reacted with various amounts of immobilized EBV, CMV, or R-MuLV (top panel) or with various amounts of immobilized C3d,g or ovalbumin (bottom panel). In both assays, CR2 binding was detected by incubation with biotinylated HB-5 MAb and streptavidin horseradish peroxidase.

by the MAbs. The anti-CR2 MAbs did not react with uncoated wells or with wells coated with BSA.

A dot blot immunoassay was used to analyze the functional reactivity of the purified 145-kDa protein. The 145-kDa protein reacted with purified EBV in a dose-dependent manner but did not react with equivalent amounts of CMV or R-MuLV (Fig. 3). The 145-kDa protein, after binding to EBV, was not detected by an irrelevant MAb, anti-B1 (data not shown). The 145-kDa protein also reacted with immobilized C3d,g but not with ovalbumin, a protein with similar physiochemical properties (Fig. 3). The specificity of EBV and C3d,g binding to the 145-kDa protein was further demonstrated by inhibition experiments. Preincubation of the purified receptor with MAb OKB7 before addition to the blots abrogated binding to EBV and to C3d,g (Fig. 4, lane 2) while AB-1, another anti-CR2 MAb, failed to block these reactions (Fig. 4, lane 3). These findings are in accord with other studies showing that OKB7, but not AB-1, blocks EBV and C3d binding to intact B cells (13; M. F. E. Siaw, G. R. Nemerow, and N. R. Cooper, submitted for publication).

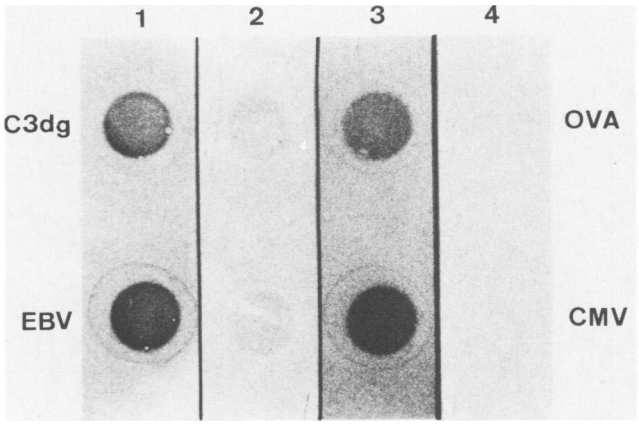

FIG. 4. Inhibition of receptor binding by anti-receptor MAb. A total of 2 μg of purified C3d,g (lanes 1 through 3, upper panel) or ovalbumin (lane 4, upper panel) or 10 ng of EBV (lanes 1 through 3, lower panel) or CMV (lane 4, lower panel) was coated onto nitrocellulose. The blots were then incubated with 40 ng of CR2 alone (lanes 1 and 4, upper and lower panels), with CR2 which had been preincubated with 6 μg of OKB7 MAb (lane 2, upper and lower panels), or with AB-1 MAb (lane 3, upper and lower panels). Reactivity was detected by the biotin-avidin system.

These studies present a high-yield, rapid purification procedure for the 145-kDa B-cell membrane protein which serves as a receptor for EBV and for C3d. The yield of the 145-kDa protein, 50 μg per 10^{10} Raji cells, represents a recovery of 75% based on 3×10^4 receptors per Raji cell (16). This is similar to results obtained from normal tonsil B cells in a recently published method which employs a combination of anion exchange and affinity chromatography on C3d,g (9). The present method is more rapid and does not require the additional purification of large amounts of C3d,g. The purification procedure contains several unusual features which we found to be important. Among these are the following: the use of Brij 96 together with DOC, a considerable improvement over Nonidet P-40 and either reagent alone; covalent cross-linking of the MAb to the column via its Fc domain; and elution of the receptor with diethylamine, which led to less denaturation than other methods attempted. The purified molecule retains the OKB7, HB-5, and AB-1 antigenic determinants and exhibits specific binding to isolated EBV as well as to C3d,g. These findings with

the purified receptor provide an unequivocal demonstration of the dual receptor functions of this 145-kDa B-cell protein.

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