# EPSTEIN-BARR VIRUS REGULATES ACTIVATION AND PROCESSING OF THE THIRD COMPONENT OF COMPLEMENT

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Epstein-Barr virus (EBV), a human herpesvirus, is responsible for, or closely associated with a number of human diseases, including infectious mononucleosis, nasopharyngeal carcinoma, Burkitt's lymphoma, an X-linked lymphoproliferative syndrome, and several autoimmune diseases (1-6). EBV is oncogenic in subhuman primates (7); in vitro it transforms normal human B lymphocytes, thereby generating immortal polyclonal B lymphoblastoid cell lines (8). EBV exhibits a highly restricted cell tropism; in vitro it only infects B lymphocytes (9-11) and nasoepithelial cells (12, 13) and only these cell types from patients contain the viral genome. Infection is initiated by binding of EBV to CR2, the 145-kD B lymphocyte receptor for the C3dg fragment of C3, the third complement component (14-17).

EBV exhibits several close associations with the complement system. First, EBV uses CR2, a complement receptor to attach to and infect B lymphocytes (14-17); second, the gp350 envelope glycoprotein of EBV shares amino acid sequence homology with C3dg (18); third, lymphoblastoid cells carrying the EBV genome in a latent state activate the alternative complement pathway (19); and, fourth, gp350, intact EBV, and EBV-producing cells also activate the alternative complement pathway (20, 21).

During complement activation by either pathway, C3 and C4 are cleaved into two fragments, C3a and C3b, and C4a and C4b, respectively. A proportion of the C3b and C4b generated in such reactions becomes covalently bound to the activator. In the case of several viruses, including EBV (22), this protein coating may neutralize infectivity (reviewed in reference 23). In addition, C3b- and C4b-coated particles bind to CR1 complement receptors that are found on phagocytes, monocytes, erythrocytes, B lymphocytes, and other cells (reviewed in reference 24). Such attachment to CR1 on cells via a C3b or C4b bridge has various functional consequences. In the case of EBV with attached C3b or C4b, the virus is redirected to CR1 rather than the virus receptor (23), and is phagocytosed if redirected to CR1 on phagocytic cells (23).

Activator bound C3b may be cleaved by a plasma enzyme, factor I, to yield iC3b

which interacts with another complement receptor, CR3, found on phagocytic cells (24, 25) (Fig. 1). Further cleavage of iC3b by factor I yields C3c (140 kD) which is released into the fluid phase, and C3dg (41kD) which remains covalently attached to the activator (26–28). Additional proteolytic cleavage of C3dg to C3d (35 kD) can be mediated by certain proteases (28). Activator-bound C3dg or C3d interact with other receptors termed CR2, found on B lymphocytes (15, 29) and CR4, present on phagocytic cells and platelets (30, 31). Activator-bound C4 is cleaved by factor I successively into iC4b and C4c and C4d (32, 33). These fragments have not been reported to interact with CR3, CR2, or CR4.

Factor I requires the participation of cofactor proteins to cleave C3b, iC3b, C4b, and iC4b. The serum proteins factor H (34) and C4 binding protein (C4bp)[1] (35), and the cell membrane proteins CR1 (26, 27, 36), CR2 (37), and membrane cofactor protein (MCP) (38) all act as cofactors for one or more I-mediated cleavage reactions. Many of the proteins with cofactor activity also regulate the activation and processing of C3 by accelerating decay-dissociation of the C3 activating enzymes of the alternative and classical pathways (39–42). An additional cell membrane protein, decay-accelerating factor (DAF), lacks cofactor activity but regulates C3 activating enzymes of both pathways (43, 44).

Certain of these regulatory activities are possessed by glycoprotein C of two herpesviruses, HSV-1 (45–47) and HSV-2 (48). In the course of examining the mechanism of complement activation by EBV, we obtained evidence that EBV acted as a cofactor for factor I–mediated cleavage of C3b. The results of these and further studies indicate that EBV possesses factor I cofactor activity for not only the cleavage of C3b, but also iC3b, C4b, and iC4b. EBV also accelerates the decay of the alternative pathway C3 convertase.

## Materials and Methods

*Viruses.* EBV was purified from supernatants of 12-0-tetradecanoylphorbol-13-acetate (TPA)-treated B95-8 and P3HR1 cells by gradient ultracentrifugation as previously described (49). The final virus preparations had a concentration of ~5 × $10^{11}$ particles/ml as determined by quantitation of DNA. Human cytomegalovirus (HCMV) was purified as described (50), and the amount of infectious virus was determined by a standard plaque assay (51).

*Complement Components and Related Reagents.* Marmoset erythrocytes were obtained through the courtesy of Dr. Suzette Tardiff, Marmoset Research Center, Oakridge Associated University, Oakridge, TN.

C3 was purifed by the method of Hammer et al. (52) with immunoadsorption used to remove albumin, C5, IgG, and IgA. C3 and C4 were radioiodinated with $^{125}$I–sodium iodide and Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions to a sp act of 2–4 × $10^5$ cpm/μg. Factors B, D, H, I, and C4bp were kindly provided in purified form by Drs. Leslie Leonard and Hans Müller-Eberhard, Research Institute of Scripps Clinic and Bernhard Nocht Institute, Hamburg, Federal Republic of Germany, respectively. Properdin was purified from the euglobulin of normal human serum by chromatography on QAE-Sephadex A25 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.15 M Tris buffer (pH 9.1, 4 mS). The breakthrough fractions were pooled, concentrated, and passed over protein A-Sepharose (Pharmacia Fine Chemicals) followed by specific immunoadsorbent columns to remove C1q and IgG. Human C4 was purified as described (52). Human C2 was purchased from Diamedix Corporation, Miami, FL.

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[1] *Abbreviations used in this paper:* C4bp, C4 binding protein; DAF, decay-accelerating factor; GVB, 1% gelatin, veronal-buffered saline; HCMV, human cytomegalovirus; MCP, membrane cofactor protein.

For assays of C3 cofactor activity, EAC142, prepared with human components and oxidized human C2, were incubated for 20 min at 37°C with $^{125}$I-C3 (100 µg/5 × 10$^8$ cells), washed and suspended in GVB (0.1% gelatin, veronal-buffered physiological saline, pH 7.4) at 10$^9$ cells/ml. These EAC1423b were converted to EAC142iC3b cells by incubating for 30 min at 37°C with 80 µg of factor I and 70 µg of factor H/10$^9$ cells. The cells were then washed and resuspended in GVB. For assays of C4 cofactor activity, sensitized sheep E were sequentially incubated with human C1 and $^{125}$I-C4 (150 µg/10$^9$ cells), washed, and suspended in GVB at 10$^9$ cells/ml. For rosetting assays, EAC142, EAC1423b, and EAC142iC3b were prepared as above with the substitution of unlabeled human purified C3 for the $^{125}$I-C3. Also, portions of the EAC142iC3b cells were converted to EAC1423d by incubation at 10$^9$ cells/ml for 30 min at 37°C with trypsin-TPCK (Worthington Biochemical Corp., Freehold, NJ) (20 µg/ml) in GVB. After the incubation, the trypsin was neutralized by the addition of excess soybean trypsin inhibitor and the cells were washed extensively.

For assays of decay-accelerating activity, EAC142 were prepared with limiting amounts of nonoxidized C2 calculated to yield ~0.5–1 hemolytic sites per cell. EAC3bBb were prepared as follows: Native C3 was incubated with sheep erythrocytes (100 µg C3 for 10$^9$ E) and 5 µg trypsin-TPCK in a total volume of 100 µl. The buffer used throughout this preparation was GVB. The cells were incubated at 23°C for 5 min and then washed thoroughly. EC3b were incubated in a final volume of 100 µl with 20 µg of factor B, 0.5 µg of factor D, and either 2 mM NiCl$_2$ or 7.5 µg of native properdin together with 2 mM MgCl$_2$ to stabilize the convertase.

*Antibodies.*   mAbs to a common class I HLA determinant, W6-32 (American Type Culture Collection, Rockville, MD), HLA-DQ and HLA-DR (Becton Dickinson & Co., Sunnyvale, CA), and B1 (Coulter Immunology, Hialeah, FL) were obtained commercially. Monoclonal (3D9) and polyclonal anti-CR1 were kind gifts of Drs. Eric Brown (Washington University, St. Louis, MO) and Brian Tack (Research Institute of Scripps Clinic) respectively. Other mAbs to CR1 were purchased from Becton Dickinson & Co. (44D) and Accurate Chemical Co., Hicksville, NY (833M), respectively. mAbs specific for C3b, C3c, and C3d were kindly provided by Dr. Hans Müller-Eberhard, Bernhard Nocht Institute, Hamburg, Federal Republic of Germany.

*SDS-PAGE and Autoradiography.*   SDS-PAGE analyses were performed under reducing conditions using the Laemmli buffer system on 10% gels or on 5–15% gradient gels (53). Samples were boiled for 3 min before electrophoresis. Kodak X-Omat RP film was used for autoradiography.

*FACS Analysis.*   Pellets of 10$^6$ Raji, B95-8, P3HR1, and T1 human lymphoblastoid cells as well as TPA-induced B95-8 cells were washed and resuspended on ice in 50-µl volumes of the mAbs to be analyzed. Quantities of the monoclonals used were as follows: Anti-HLA-DQ and DR and B1 were used undiluted; mAb 3D9 (anti-CR1, prepared from ascites with octanoic acid) was used at 5–20 µg/ml as specified; mAb W6-32 (anti-MHC class I clone) was used at a 1:4 dilution; mAb 44D (anti-CR1, Becton Dickinson & Co.) was used undiluted; mAb 833M (Accurate Chemical Co.) was used at a 1:10 dilution. All of the monoclonals were centrifuged for 10 min at 10,000 *g* before use. The mAbs were incubated with the cells for 1 h on ice and subsequently washed with ice cold PBS-BSA. The pellets were then resuspended in 50 µl of 1:10 diluted FITC-labeled affinity-purified goat anti–mouse IgG (γ chain specific; Kirkegaard & Perry Laboratories, Gaithersburg, MD) and incubated for 30 additional minutes on ice. The cells were washed and resuspended in 0.5 ml of PBS-BSA. Fluorescence analyses were performed with a FACS (model 440; Becton Dickinson & Co.) equipped with a 5 W argon laser operating at 488 nm, 0.3 W. Fluorescence parameters were collected after gating for a combination of forward light scatter and orthogonal light scatter (side scatter), used to discriminate viable from nonviable cells. Data were stored in list mode and analyzed using the Becton Dickinson & Co. Consort 30 FACS data analysis software package.

*Cofactor Activity Assays.*   The assay for cofactor activity for the factor I–mediated cleavage of C3b and iC3b has been previously described (26). Briefly, 10$^7$ EAC1423b or EAC1423bi prepared with $^{125}$I-C3 were incubated for 30 min at 37°C in a total volume of 100 µl GVB with factors to be tested for cofactor activity. Factors H and I in amounts indicated in the

text, 40 µl B95-8 or P3HR1-derived EBV or HCMV and $10^7$ washed human erythrocytes were added to $10^7$ C3-bearing erythrocytes in various combinations. 80 µg of 3D9 mAb to CR1 was also included in certain samples, as indicated in the text. The cells were centrifuged for 2 min at 12,000 g and the supernatants were removed and analyzed by SDS-PAGE under reducing conditions followed by autoradiography. In some experiments, the cell pellets were also extracted with SDS and analyzed by SDS-PAGE under reducing conditions. Quantities of factors to be tested for cofactor activity for the factor I-mediated cleavage of C4b were as follows for $10^7$ cells: 20 ng factor I, 20 µg C4bp, and 40 µl EBV, in various combinations.

*Decay-accelerating Activity Assays.*    Both classical and alternative pathway C3 convertase decay rates were measured by mixing sheep E bearing the appropriate enzyme with the desired concentration of either purified virus, control buffer, or known DAF. For each time point in the assay, $10^7$ sheep E in 10 µl were combined with 40 µl of the factor under study, on ice, and then warmed to 30°C quickly in a circulating water bath when timing began. When marmoset or human erythrocytes were assessed for decay-accelerating activity, $10^8$ primate erythrocytes were included in the 40 µl. 80 µg of mAb to CR1 (3D9) was also included, where specified. At timed intervals, samples of 50 µl were removed and residual convertase activity was measured by the addition of 1 ml guinea pig serum diluted 1:50 in GVB containing 20 mM EDTA. After further incubation for 20 min at 37°C, the percent hemolysis was calculated and expressed as the reciprocal of the negative natural logarithm of the number of hemolytic sites per cell: [-ln(1-y)].

*C3 Binding Assays.*    Rosetting of lymphoid cells with sheep erythrocytes bearing complement intermediates was measured as follows. $5 \times 10^5$ TPA-treated virus-expressing B95-8 or P3HR1 cells, CR1-bearing T1 human lymphocytes transformed with B95-8 virus and carried as a lymphoblastoid cell line, or human peripheral blood monocytes (freshly isolated by Ficoll-Hypaque gradient followed by elutriation) were incubated with $5 \times 10^7$ complement-bearing sheep erythrocytes (EAC142, EAC1423b, EAC142iC3b, or EAC1423d) in a total volume of 100 µl. After mixing, the cells were briefly pelleted (100 g, 3 min) and the cell pellets were incubated without mixing for 1 h at 37°C. The cell pellets were gently resuspended and rosetting was evaluated on a Zeiss phase contrast microscope. Cells with four or more red cells attached to their surface were considered rosette-positive.

The dot blot immunoassay has been previously described (18, 54). Briefly, 10 µg of native C3, C3b, C3dg, or C4 were immobilized on nitrocellulose paper. After blocking and wash steps, paper strips containing the blots were incubated at 23°C for 60 min with 50 µl of purified B95-8-derived EBV or TRIS/dextran virus gradient buffer as a negative control diluted to 2.5 ml in wash buffer. The reagents to detect binding were a biotinylated mAb to the membrane gp350 antigen of EBV (designated BOS-1) (18) streptavidin-horseradish peroxidase (HRP) (Amersham Corp., Arlington Heights, IL) and 2,2'-azinolois(3-ethylbenthiazoline sulfonic acid) (ABTS) chromogenic substrate (250 µg/ml ABTS, 0.03% $H_2O_2$ in citrate buffer, pH 4.5). The assay was also performed in the reverse direction, with 2.5 µl of purified EBV or virus gradient buffer immobilized on nitrocellulose paper followed, after blocking and washing by 10 µg of the complement fragments. The detection system for this assay used rabbit antisera to the complement fragments, biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) streptavidin-HRP and ABTS substrate.

The same type of assay was performed using a previously described ELISA (18, 54) with slight modifications. Microtitration plates were coated with either 5 µl of purified B95-8-derived EBV, HCMV, or control buffer diluted to 50 µl with bicarbonate coating buffer. This coating step proceeded for 18 h at 37°C in an uncovered plate, to allow dessication onto the plate. This step has been found to increase the sensitivity of the assay. After blocking and washing, 10 µg/well of complement fragments were incubated in the wells, in duplicate, at 23°C for 1 h. The antibodies used for detection were mAbs to C3b, C3c, and C3d mixed together. The remainder of the assay was carried out as described earlier.

Direct binding assays to assess EBV attachment to complement-coated erythrocytes were also performed. $5 \times 10^7$ EACs in 50 µl were incubated in duplicate with 50 µl of purified EBV (B95-8) for 30 min at 37°C with occasional mixing. The cells were washed twice in PBS containing 0.2% BSA (PBS/BSA) and the cell pellets were then resuspended in purified mAb to EBV gp350 (BOS-1) at a concentration of 10 µg/ml. Incubation proceeded at 37°C

for 30 min followed by two washes. The cell pellets were then incubated with 50 μl of bi-otinylated goat anti–mouse IgG, diluted 1:1,000 (Kirkegaard & Perry Laboratories, Inc.) for 30 min at 37°C. After washing, the pellets were resuspended in 50 μl of ^{125}I-streptavidin (Amersham Corp.) diluted 1:20 in PBS/BSA. Incubation proceeded at 37°C for 30 min after which the cells were washed thoroughly, transferred to clean tubes, and counted in a gamma counter (model 5260; Packard Instrument Co., Downers Grove, IL).

## Results

*C3c Is Generated During the Incubation of EBV with Serum.*     In the course of our studies of complement activation by EBV (21), we observed that cleavage products not only characteristic of iC3b, as expected, but also of C3c (Fig. 1), appeared during the incubation of purified B95-8 strain EBV with immune human serum containing ^{125}I-C3 (Fig. 2, lane 1). While minor amounts of the 68-kD and 46-kD C3 fragments characteristic of fluid phase cleavage of C3b into iC3b were found, the most striking feature was the presence of a 25-kD C3 fragment. The size and marked radiolabeling of this fragment are consistent with the NH2-terminal α chain fragment of C3c. Also observed were traces of a 43-kD fragment that could represent the other α' chain fragment or C3dg, which has a molecular weight of approximately this size and is poorly radiolabeled by the procedure used. In the control (Fig. 2, lane 2), only intact C3 and minor traces of iC3b (68- and 46-kD bands) were seen.
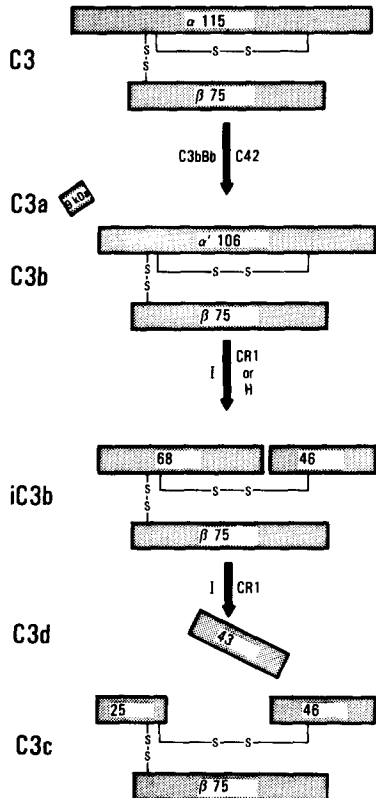


FIGURE 1.   C3 activation and processing fragments. Apparent molecular weights ($\times$ $10^{-3}$) from SDS-PAGE analyses are shown. Conceptual model is from reference 65.
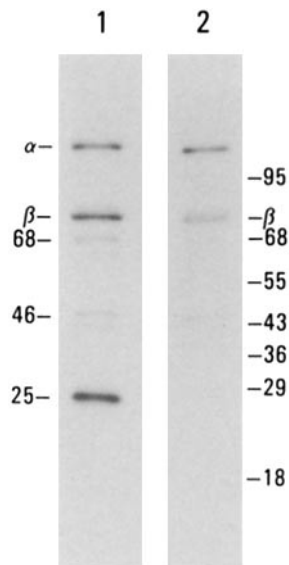
FIGURE 2. C3c is generated during the incubation of EBV with serum. EBV (B95-8) was incubated with normal immune human serum (lane $1$) or with EDTA-chelated normal immune human serum (lane $2$) supplemented with $^{125}$I-C3 for 30 min at 37°C followed by SDS-PAGE analysis and autoradiography under reducing conditions. C3 $\alpha$ (110-kD) and $\beta$ (75-kD) chains are indicated. The migration positions and molecular masses (kD) of the marker proteins are shown on the right; the molecular masses of the observed bands are indicated on the left.

Although human serum contains cofactors for the factor I–mediated cleavage of C3b to iC3b (factor H and C4bp), it lacks cofactors for the factor I–mediated cleavage of iC3b into C3c and C3dg. Thus the appearance of a C3 fragment characteristic of C3c after incubation of EBV with serum suggested to us that EBV either possessed the ability to degrade C3b and iC3b, or acted as a cofactor for their breakdown. These aspects were investigated in the following experiments.

*EBV Is a Cofactor for the Factor I–mediated Cleavage of C3b.* To determine whether purified EBV either possessed the ability to directly cleave C3b or acted as a cofactor for C3b degradation by factor I, EAC142 $^{125}$I-C3b were incubated for 30 min at 37°C with buffer or with various combinations of factor I, factor H, purified EBV, or human erythrocytes (as a source of CR1). After centrifugation, the supernatants were analyzed by SDS-PAGE under reducing conditions followed by autoradiography. As shown in Fig. 3, supernatants of cells incubated with B95-8-derived EBV together with factor I revealed the presence of the 106-kD $\alpha'$ and 75-kD $\beta$ chains indicative of the presence of C3b as well as a 68-kD fragment characteristic of the fluid phase cleavage of the $\alpha'$ chain C3b to generate iC3b (lane $5$). Since the EAC1423 were well washed before incubation with buffer or the other reagents, the presence of C3b and iC3b in the supernatant is most likely explained by spontaneous elution of nonspecifically bound C3b from the cells and its degradation in the fluid phase. Others have also observed degradation products characteristic of iC3b in similar mixtures of factor I, cofactors, and immune complex–bound C3b and interpreted these findings as indicating release and degradation of noncovalently bound C3b (26). The 68-kD fragment was also observed in supernatants of mixtures containing factors I and H (lane $3$) and factor I and human E as a source of CR1 (lane $4$), both of which served as positive controls. The factor I–human E mixture also generated a 25-kD fragment, which is characteristic of C3c. The 46-kD $\alpha'$ chain fragment of iC3b is poorly labeled by the method used and thus not seen in the autoradio-
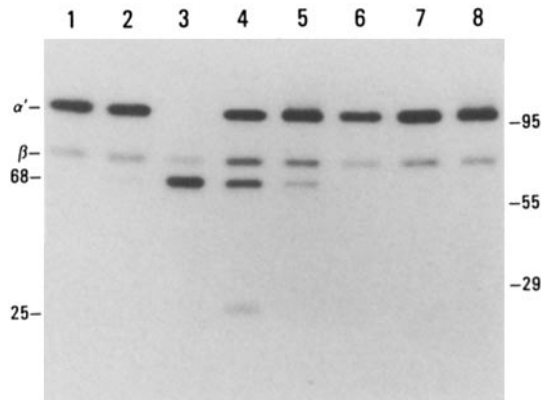
FIGURE 3.    EBV is a cofactor for the factor I–mediated cleavage of C3b. SDS-PAGE analysis (reducing conditions) of $^{125}$I-C3 released from EAC1423b by incubation for 30 min at 37°C with GVB (lane *1*); factor I alone (lane *2*); factor I and factor H (lane *3*); factor I and human E (lane *4*); factor I and EBV (B95-8) (lane *5*); factor H alone (lane *6*); human E alone (lane *7*); EBV (B95-8) alone (lane *8*). 2 µg of factors H and I, 40 µl of EBV, or 10$^7$ human erythrocytes were used.

graph. Supernatants of cells incubated with buffer (lane *1*) or with EBV (lane *8*), factor H (lane *6*) or human E (lane *7*) alone showed no cleavage of the C3b α′ chain; a trace of the 68-kD fragment was seen in the supernatant of cells incubated with factor I alone (lane *2*). Qualitatively identical results were obtained in two additional experiments of this type with two other B95-8 EBV preparations (not shown), as well as in four experiments with P3HR1-derived EBV (see last section of Results).

In addition to analyzing the supernatants for the presence of C3 derivatives, cell-bound C3 fragments were also examined in a number of these studies. Mixtures containing EAC3b together with factor I and either human E or EBV showed the presence of a 68-kD band characteristic of iC3b (not shown). Since a 68-kD band was not found on cells from mixtures containing EAC3b together with either buffer, factor I, human E, or EBV alone, its presence documents the generation of iC3b from noncovalently bound C3b on the cell surface by factor I acting with either human E or EBV as a cofactor. In addition, EAC3b from all of the reaction mixtures showed the presence of variable amounts of multiple high molecular mass bands (>100 kD) reflecting the covalent attachment of the C3 α′ chain, or its fragments to multiple erythrocyte membrane constituents. Small amounts of lower molecular weight bands were also found. Identification of specific bands in these gels was not possible due to the complexity of the patterns.

These above data cumulatively indicate that EBV does not directly degrade C3b but rather acts as a cofactor for the factor I–mediated cleavage of fluid phase or cell-bound C3b into iC3b. Because of the inability to identify specific cleavage products of C3 on the cell due to covalent and noncovalent α′ chain fragment binding to membrane constituents, as well as the inefficient radiolabeling of C3dg and of the COOH-terminal fragment of iC3b, we chose to analyze complement fragments in the supernatants in subsequent studies.

*EBV Is a Cofactor for the Factor I–mediated Cleavage of iC3b.*    To determine whether EBV also possessed the ability to either degrade iC3b, or alternatively, acted as a cofactor for iC3b degradation, EAC142 $^{125}$I-iC3b were incubated for 30 min at 37°C with buffer or with various combinations of B95-8-derived EBV and factor I. HCMV, another human herpesvirus, was used as a control. After centrifugation the supernatants were analyzed by SDS-PAGE followed by autoradiography. As shown in Fig.
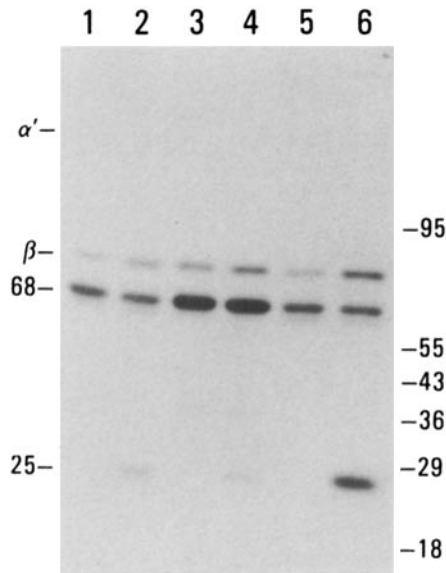
FIGURE 4. EBV is a cofactor for the factor I-mediated cleavage of iC3b. SDS-PAGE analysis (reducing conditions) of $^{125}$I-iC3 released from EAC142iC3b by incubation with GVB (lane *1*); factor I alone (lane *2*); HCMV alone (lane *3*); HCMV and factor I (lane *4*); EBV (B95-8) alone (lane *5*); EBV (B95-8) and factor I (lane *6*). 2 µg of factor I or 40 µl of EBV or HCMV were used.

4, supernatants of cells incubated with EBV together with factor I revealed the presence of a 25-kD fragment, consistent with the NH₂-terminal α' chain fragment of C3c (lane *6*). The apparent molecular weight of the released C3 fragment, determined by SDS-PAGE analyses under nonreducing conditions, was 139 kD (not shown). This is consistent with the value of 146 kD obtained by adding the molecular weights of the NH₂- and COOH-terminal α' chain fragments of C3c (25 kD and 46 kD, respectively) together with that of the β chain (75 kD). Much smaller amounts of this fragment were released by factor I alone (lane *2*) and none was released spontaneously (lane *1*) or by EBV alone (lane *5*). HCMV did not show such activity (lanes *3* and *4*). Similar results were obtained in four additional studies of this type with three different preparations of B95-8-derived EBV. These studies indicate that EBV is also a cofactor for the factor I-mediated cleavage of iC3b.

*EBV Is a Cofactor for the Factor I-mediated Cleavage of C4b and iC4b.* Similar studies to those described above were carried out with EAC1 $^{125}$I-C4b in order to ascertain whether EBV was also a cofactor for the factor I-mediated cleavage of C4b. As shown in Fig. 5, supernatants of cells incubated with EBV (B95-8) together with factor
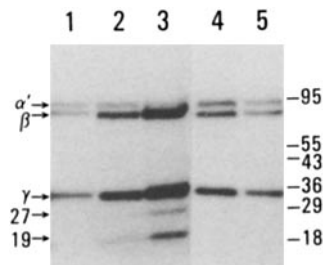


FIGURE 5. EBV is a cofactor for the factor I-mediated cleavage of C4b. SDS-PAGE analysis (reducing conditions) of $^{125}$I-C4 released from EAC14 by incubation with factor I alone (lane *1*); factor I and C4-binding protein (lane *2*); factor I and EBV (B95-8) (lane *3*); C4bp alone (lane *4*); EBV (B95-8) alone (lane *5*). 20 ng of factor I, 20 µg of C4bp or 40 µl of EBV were used.
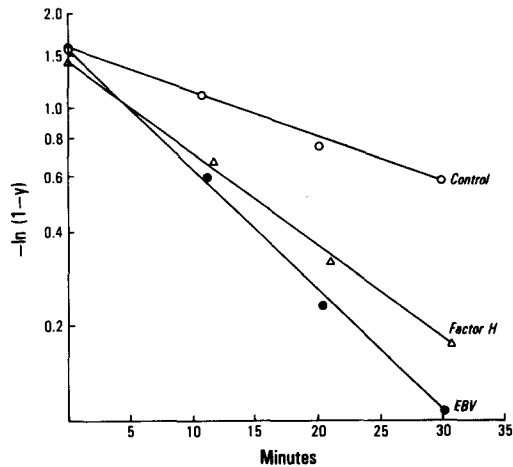
FIGURE 6. EBV accelerates the decay of the nickel-stabilized alternative pathway C3 convertase. Sheep E bearing the nickel stabilized alternative pathway C3 convertase, C3b, Bb, were incubated at 30°C with buffer, EBV (B95-8), or factor H. Periodic samples were incubated with EDTA guinea pig serum as a source of C3-C9, after which lysis was assessed and expressed as $-\ln(1 - y)$.

I revealed the presence of only a trace of the 89-kD α' chain together with the 77-kD and 35-kD β and α chains, respectively (lane 3). In addition, two fragments with molecular masses of 27 kD and 19 kD (lane 3), consistent with the presence of C4c, were observed. The same fragments were observed in mixtures containing C4bp and factor I (lane 2). These fragments were not released by factor I alone (lane 1), C4bp alone (lane 4), or EBV alone (lane 5). Similar results were obtained in two other studies of this type. These studies indicate that EBV is also a cofactor for the factor I–mediated cleavage of both C4b and iC4b.

*EBV Accelerates the Decay of the Alternative, but not the Classical Pathway C3 Convertase.* A common property of several of the factor I cofactors is their ability to accelerate decay dissociation of the alternative and/or classical pathway C3 convertases. As shown in Fig. 6, B95-8-derived EBV reduced the half-life of the nickel-stabilized alternative pathway C3 convertase, C3bBb, on sheep E from 21.5 to 7.8 min. Similar accelerated decay was observed in the presence of factor H. Accelerated decay of the nickel- or properdin-stabilized alternative pathway C3 convertases in the presence of EBV was found in seven additional studies with four different preparations of
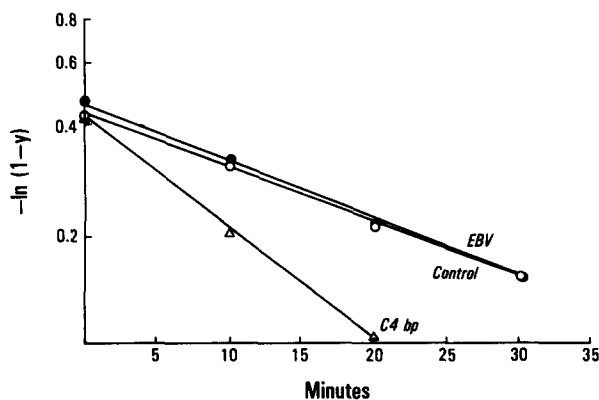


FIGURE 7. EBV does not accelerate the decay of the classical pathway C3 convertase. EAC142 prepared with human components and nonoxidized C2 were incubated with buffer, EBV (B95-8), or C4bp at 30°C. Periodic samples were incubated with EDTA guinea pig serum as a source of C3-C9, after which lysis was assessed and expressed as $-\ln(1 - y)$.

B95-8-derived EBV. Qualitatively identical results were obtained in two studies of this type carried out with P3HR1-derived EBV.

In contrast, the same amount of EBV (B95-8) did not influence the decay rate of the classical pathway C3 convertase, C4b2a, on sheep E as depicted in Fig. 7. Similar results were obtained in two other studies of this type. However, C4bp reduced the half-life of the classical pathway C3 convertase from 20 to 10 min (Fig. 7). Thus, EBV accelerated the rate of decay of the alternative, but not classical pathway C3 convertase.

*EBV Does Not Readily Bind to C3b.*    The above approaches indicated that EBV interacted with factor I to facilitate C3b cleavage and also produced alternative pathway decay acceleration, likely also via an effect on C3b. Some, although not all such cofactors and decay accelerators, bind C3b (see Fig. 12). Several approaches were used to assess the potential C3b binding ability of EBV. First, the possible ability of EAC3b to form rosettes with TPA-treated B95-8 and P3HR1 cells was examined in several experiments. No rosetting of the virus-expressing cells (in 300 lymphoid cells) with EAC3b was obtained although all of the CR1-bearing T1 human lymphoblastoid cells and most of the monocytes tested in the same experiments formed large rosettes (greater than six erythrocytes per leukocyte) with EAC3b. In a second approach, EBV (B95-8) was incubated with C3b (and other C3 fragments) that had been immobilized on nitrocellulose. Subsequent incubation with a biotinylated mAb to EBV followed by streptavidin HRP and substrate failed to provide evidence for EBV binding. This test system readily detected EBV directly bound to the nitrocellulose. The same assay performed with C3 fragments bound to the wells of microtitration plates also failed to provide evidence for binding of EBV to C3b. This test carried out at one-third normal ionic strength also did not provide evidence for EBV binding. These assays were also performed in the reverse direction, i.e., by incubating C3b and other C3 fragments with B95-8-derived EBV immobilized on either nitrocellulose or plastic microtitration plate. Rabbit antibody to C3 followed by biotinylated goat anti–rabbit Ig, streptavidin HRP, and substrate were added sequentially. These experiments also failed to provide evidence for EBV binding to C3b. Finally, purified EBV (B95-8) was incubated with EAC3b. Possible binding of EBV was assessed by incubation with an mAb to EBV followed by biotinylated goat anti–mouse Ig, and $^{125}$I-streptavidin. The cells after washing were examined for bound radioactivity. These studies did not provide evidence for EBV binding to cell bound C3b. We conclude that

TABLE I

*Marmoset Erythrocytes Do Not Accelerate C3bBb Decay*

| Erythrocytes | $T_{\frac{1}{2}}$ |
|---|---|
| | *min* |
| Control* | 22.5 |
| Marmoset E | 21.5 |
| Marmoset E + 3D9 | 22.5 |
| Human E | 14 |
| Human E + 3D9 | 20.5 |

* EAC3bBb were incubated at 30°C with human or marmoset erythrocytes in the presence or absence of 3D9 anti-CR1 mAb.
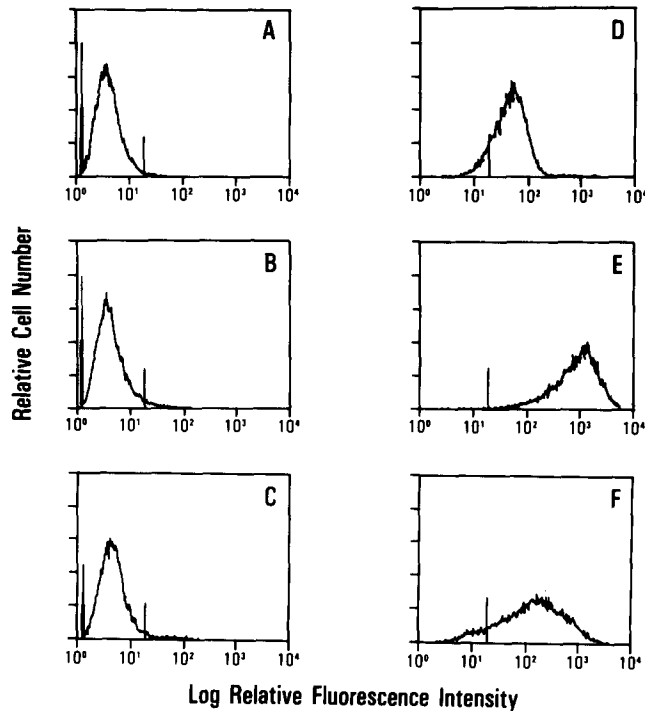
FIGURE 8. B95-8 cells do not bear CR1. FACS analysis of the reactivity of B95-8 cells with various monoclonal antisera directed against human B cell antigens. (*A*) Control. Reactivity with anti-HLA-DQ (*B*), anti-CR1 (3D9) (*C*), HLA class I (*D*), HLA-DR (*E*) and anti-B1 (*F*) are shown.

the interaction of EBV with C3b, although functionally significant, is too weak or transitory to be directly demonstrated.

*C3/C4 Cofactor and Decay-accelerating Activities Are Not Due to Cr1 Associated with or Bound to EBV.* Several approaches were used to determine whether the observed C3 regulatory activities were due to CR1 acquired from the B95-8 or P3HR1 cell lines during cellular disruption or EBV maturation. As described in the previous section, TPA-treated B95-8 cells failed to form rosettes with EAC3b. This very sensitive assay is the standard functional test for CR1.

Since many of the assays were carried out with EBV derived from the B95-8 cell line, which is of marmoset origin, we sought to determine whether marmoset CR1, if it should be present in EBV derived from the cell line, would crossreact functionally with human complement components. We assessed marmoset erythrocytes, as a source of marmoset CR1, for ability to accelerate decay of the C3bBb convertase since this is not only a sensitive test system for CR1, but also, marmoset cell–derived EBV possesses this activity. However, as shown in Table I, marmoset erythrocytes did not accelerate C3bBb decay while the positive control, human E, did so. The latter activity was also inhibited by the 3D9 mAb to CR1, as anticipated. Qualitatively similar results were obtained in two other assays of this type with erythrocytes from two marmosets. Thus marmoset CR1 is not reactive with human C3bBb.

We also examined marmoset B95-8 cells, as well as human P3HR1 cells, for the presence of immunochemically reactive CR1 by FACS. As shown in Fig. 8 *C*, the 3D9 mAb to CR1 failed to react with marmoset B95-8 cells. Several other monoclonal antisera directed against human B cell antigens were also examined. As shown,
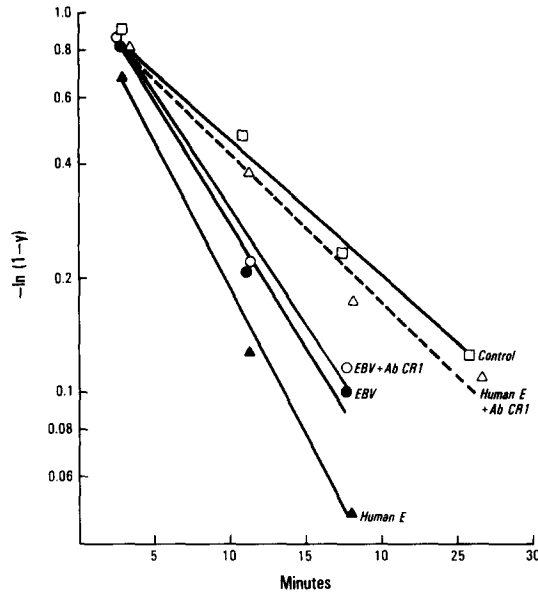
FIGURE 9. Anti-CR1 does not block the alternative pathway C3 convertase decay accelerating activity of EBV. Sheep E bearing the nickel-stabilized alternative pathway C3 convertase, C3bBb, were incubated at 30°C with buffer, B95-8 EBV, B95-8 EBV + anti-CR1, human E as a source of CR1 and human E + anti-CR1. Samples were processed as described in the legend to Fig. 6.

good reactivity of the marmoset cells with antibody to a human common HLA class I determinant (panel *D*), to human HLA-DR (panel *E*), and to the B1 antigen (panel *F*) was observed. HLA-DQ was not detected (panel *B*). Two anti-CR1 mAbs (833M and 44D) and a polyclonal antibody to CR1 also failed to detect CR1 on the B95-8 cells (not shown).

To examine the possibility that undetectable traces of immunochemically reactive CR1 possibly present on the cells might be incorporated into EBV, we assessed the ability of monoclonal anti-CR1 to inhibit the alternative pathway C3 convertase-accelerating activity of B95-8 EBV. As shown in Fig. 9, the decay-accelerating activity of B95-8-derived EBV was not altered by 3D9 anti-CR1 whereas the same amount of anti-CR1 reversed the decay-accelerating activity of CR1 on human E.

Because of uncertainties about detection of marmoset CR1, studies were carried out to assess the possible presence of human CR1 on human EBV-producing cells or EBV derived from the cells. As noted in the previous section, the P3HR1 EBV-producing cells also failed to rosette with EAC3b, the standard functional assay for CR1. Human P3HR1 cells were also examined for the presence of CR1 by FACS. As shown in Fig. 10, P3HR1 cells failed to react with either 833M or 44D mAbs to CR1 (panel *E*), whereas T1 lymphoblastoid cells reacted with both antibodies (panel *F*). B95-8 cells, as noted above, did not react with these antibodies (panel *D*). An identical pattern of reactivity or nonreactivity was observed with 3D9 mAb to CR1 (not shown). Thus human P3HR1 cells also do not bear immunochemically detectable CR1.

Although CR1 could not be detected on the virus-producing cells, we examined whether undetectable traces of CR1 might account for the results by examining the possible ability of anti-CR1 to block cofactor activity of EBV derived from the human cell line. The 3D9 monoclonal anti-CR1 examined is known to block CR1 func-
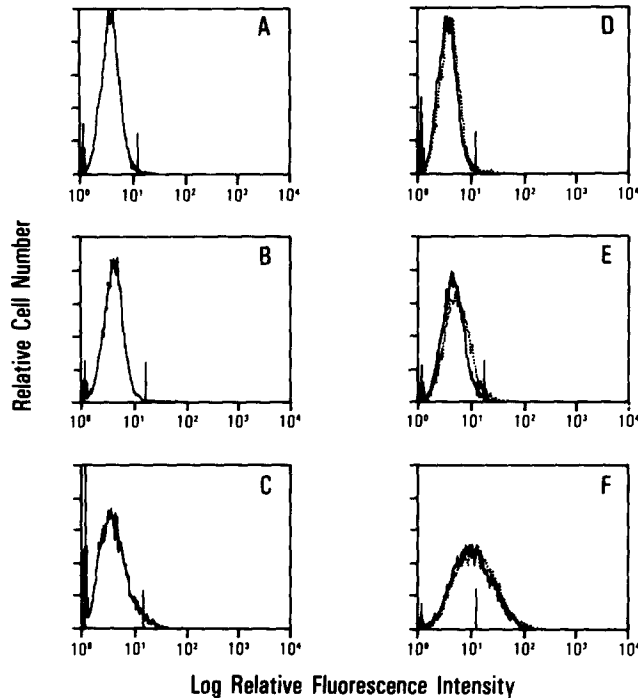
Figure 10. P3HR1 cells do not bear CR1. FACS analysis of the reactivity of B95-8, P3HR1, and T1 lymphoblastoid cells with anti-CR1 mAbs. (A, B, and C) The B95-8, P3HR1, and T1 controls. Reactivity of 833M (*solid line*) and 440 (*dotted line*) anti-CR1 mAbs with B95-8 (D), P3HR1 (E) and T1 (F) lymphoblastoid cells are shown.

**Log Relative Fluorescence Intensity**

tional activities. In these studies EAC142 $^{125}$I-C3b were incubated with various combinations of factor H, factor I, human E, P3HR1-derived EBV, and monoclonal 3D9 anti-CR1. The supernatants were analyzed by SDS-PAGE as described earlier. As shown in Fig. 11, supernatants of cells incubated with P3HR1-derived EBV together with factor I showed the presence of the 68-kD fragment characteristic of iC3b (lane 6), as also observed with B95-8 EBV (Fig. 3). The 68-kD fragment was also observed in mixtures containing factors I and H (lane 3) and factor I and human E (lane 8), but not in the control lanes (lanes 1, 2, 4, and 5). Further cleavage was
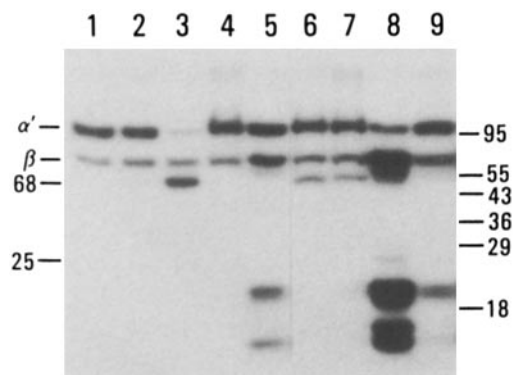


Figure 11. Anti-CR1 does not block the factor I cofactor activity of EBV for the cleavage of C3b. SDS-PAGE analysis (reducing conditions) of $^{125}$I-C3 released from EAC142 $^{125}$I-C3b by incubation for 30 min at 37°C with factor H alone (lane 1); factor I alone (lane 2); factor H and factor I (lane 3); P3HR1 derived EBV alone (lane 4); human E alone (lane 5); factor I and P3HR1-derived EBV (lane 6); factor I, P3HR1-derived EBV, and 3D9 monoclonal anti-CR1 (lane 7); factor I and human E (lane 8); and factor I, human E, and 3D9 monoclonal anti-CR1 (lane 9). 30 ng of factor I, 500 ng of factor H, 40 µl of EBV, 10$^7$ human E, and 80 µg of 3D9 antibody were used; anti-CR1 was preincubated with EBV or human E for 30 min at 37°C before addition to EAC142 $^{125}$I-C3b.

| | EBV | gC | CR1 | CR2 | DAF | MCP | H | C4bp |
|---|---|---|---|---|---|---|---|---|
| C3b Binding | - | + | + | + | - | + | + | + |
| Cofactor for Cleavage of C3b by I | + | - | + | - | - | + | + | + |
| Cofactor for Cleavage of iC3b by I | + | | + | + | - | - | - | - |
| Alternative Pathway C3 Convertase Decay | + | + | + | - | + | - | + | - |
| C4b Binding | - | - | + | | - | + | - | + |
| Cofactor for Cleavage of C4b by I | + | | + | | - | + | - | + |
| Cofactor for Cleavage of iC4b by I | + | | + | | - | - | - | + |
| Classical Pathway C3 Convertase Decay | - | - | + | - | + | - | - | + |

FIGURE 12. Activity profiles of C3 and C4 regulatory proteins.

also observed in the samples containing erythrocytes. Of considerable interest, monoclonal anti-CR1 did not block generation of the 68-kD fragment in reaction mixtures containing EAC3b, factor I, and P3HR1-derived EBV (lane 7) although it efficiently blocked generation of iC3b from C3b by factor I acting together with human E (lane 9). Similar results were obtained in another study with P3HR1-derived EBV.

The various studies described above rule out CR1 as responsible for the biological activities observed. Furthermore, the finding that marmoset B95-8 EBV as well as P3HR1 EBV derived from marmoset and human cells, respectively, possess the same complement regulatory functions strongly suggests that EBV is responsible for the functional properties.

## Discussion

A number of molecules have been identified that regulate C3b and C4b activation and processing either by acting as cofactors for factor I-dependent cleavage or by accelerating the decay of the C3 convertases (Fig. 12). We have found complement regulatory activities in purified preparations of EBV derived from virus-producing marmoset and human B lymphoblastoid cells. In the presence of factor I, EBV enhanced the cleavage of bound C3b to iC3b, iC3b to C3c and C3dg, C4b to iC4b, and iC4b to C4c and C4d. No breakdown of C3b, iC3b, or C4b by EBV was seen in the absence of factor I, indicating that the EBV preparations did not contain proteases capable of degrading these proteins. EBV also accelerated the decay of the alternative pathway, but not the classical pathway C3 convertase. This pattern of reactivity differs from that of all other reported serum, cell membrane, and viral cofactors (Fig. 12). For example, the serum proteins factor H and C4bp both act as cofactors for the cleavage of C3b to iC3b under physiological conditions (27, 28, 35) and as DAFs for the alternative pathway and classical pathway C3 convertases respectively (39, 40). The membrane glycoprotein CR1 is a cofactor for the cleavage of bound C3b and iC3b as well as C4b and iC4b (26, 27, 36), it also is a decay accelerating factor for both convertases (41, 42). The membrane protein DAF has no cofactor activity but decay accelerating activity for both convertases (43, 44). Another cell surface regulatory factor, MCP, enhances the cleavage of C3b to iC3b by factor I but lacks decay-accelerating activity for both convertases (38). Neither the activity of MCP in the cell membrane, nor its action on cell-bound C3b has as yet been examined.

The spectrum of activities of EBV is most similar to that of CR1, but differs in that EBV-expressing cells, in contrast to CR1-bearing cells, do not rosette with C3b-coated erythrocytes. This is the primary functional assay for CR1. A second distinguishing feature is its inability to accelerate the decay of the classical pathway C3 convertase. Nevertheless, multiple other studies were carried out to determine whether the observed activities might represent the actions of CR1 acquired from the virus producing cell lines. Among these, we examined whether marmoset erythrocytes, as a source of marmoset CR1, would accelerate the decay of the alternative pathway C3 convertase as do human CR1 and EBV. Marmoset erythrocytes undoubtedly bear CR1 as do other primate erythrocytes (55), and have been reported to exhibit C3b (CR1)-dependent rosetting in a somewhat unconventional test system (56). However, erythrocytes from two marmosets lacked the ability to accelerate decay of the alternative pathway C3 convertase, although human E possessed such activity and this activity was inhibited by anti-CR1. Next we assessed whether an mAb to CR1 could reverse the decay-accelerating activity of B95-8 EBV. No effect on the decay rate was noted, although the same antibody reversed the decay-accelerating activity of CR1 on human erythrocytes. We also evaluated whether CR1 could be detected on TPA-treated marmoset B95-8 cells with three mAbs to CR1 as well as a polyclonal antibody to CR1. No reactivity was observed. Since the B95-8 cell line is of marmoset origin and the antibodies are directed against human proteins, we examined multiple antisera to human cell surface antigens to determine whether cross-reactivity of the antisera with marmoset cell surface antigens is commonly observed. Good reactivity with antibody to a common HLA class I determinant, to HLA-DR, and to the human B1 antigen was observed. Although HLA-DQ was not detected on the marmoset cells, this finding must be interpreted with caution since human B cells and lymphoblastoid cells vary considerably in their expression of the two MHC class II determinants, HLA-DQ and HLA-DR.

The strong crossreactivity of antibodies to human MHC class I, HLA-DR, and B1 antigens with the equivalent antigens on the marmoset B95-8 cells coupled with the absence of reactivity with monoclonal and polyclonal antibodies to human CR1 suggested that the virus-producing marmoset B95-8 cells lack CR1. The failure of anti-CR1 to block the decay-accelerating activity of B95-8-derived EBV also implied that CR1 was not responsible for the observed activity. Finally, the studies with marmoset erythrocytes suggested that marmoset CR1, even if present, would be unable to accelerate human C3bBb decay as did EBV. Nevertheless, some uncertainty remained because of the different species involved. To definitively eliminate the possibility that CR1 in the EBV preparations was responsible for the observed activities, we carried out a number of studies with human P3HR1 EBV-producing cells and with EBV purified from the human cells.

First, we evaluated whether P3HR1 cells would rosette with C3b-bearing erythrocytes. No reactivity was observed although known CR1-bearing controls readily rosetted. Second, we assessed the presence of CR1 on the P3HR1 cells with three mAbs to CR1. No reactivity was found although the antibodies reacted with CR1 bearing, non-EBV-producing human B lymphoblastoid cells. Third, the factor I cofactor activity of purified EBV from the P3HR1-bearing cells was not blocked by the 3D9 mAb to CR1 although this antibody inhibited human E-mediated, CR1-dependent cofactor activity in the same experiments.

The above studies cumulatively indicate that CR1 is not responsible for the factor I cofactor and decay-accelerating activities exhibited by purified EBV preparations. We have not directly examined whether the observed activities are properties of EBV or of a cellular protein other than CR1 incorporated into, or attached to EBV. We feel, however, that the regulatory properties are most likely virus related for three reasons. First, the spectrum of activities differs from that of any of the known cellular or serum cofactors (Fig. 12). Second, EBV derives its envelope from the nuclear membrane, not the plasma membrane; furthermore, cellular proteins tend to be excluded from viral envelopes. Also, the virus-containing supernatant is subjected to ultrafiltration before initiating purification, and isolation is by banding in a rate zonal density gradient. Finally, and most telling, EBV preparations derived from both marmoset and human cell lines possessed the same decay-accelerating and factor I cofactor activities. It is unlikely that cells from the two species possess a principle that regulates human complement components and that is incorporated into EBV from the two cell lines. It is more probable that the functional regulatory activities are properties of a protein encoded by the viral genome. A final answer, however, must await the isolation and characterization of the factor.

Approximately $2 \times 10^9$ EBV particles possessed somewhat less factor I cofactor activity for C3b cleavage than $10^7$ human erythrocytes (Fig. 3). The human erythrocytes likely possess $\sim$1,000 CR1 per cell (57) since they were preselected for high activity. The regulatory activity of EBV is clearly less than that of CR1 in this test system, although it is within the published range of activity of other regulatory proteins. For example, MCP is more than 50 times as active as factor H in acting as a factor I cofactor for C3b cleavage (38). Factor H is 2 times more active than CR1 which in turn is 17 times more efficient than C4bp (58). The efficiency of these factors in this test system thus spans more than a 3-log range. Clearly, such quantitative assessments in nonphysiological, nonoptimized test systems do not reflect biological effectiveness in vivo. This is particularly true of EBV, which is unlikely to have evolved and maintained this trait if it did not provide a protective advantage for the virus.

For the cofactor and decay-accelerating activities of EBV to be biologically meaningful, they would have to act on C3b bound to the same viral particle because multiple EBV particles would not likely be found in close proximity in vivo. Although both CR1 (59) and DAF (44), two of the membrane-associated complement regulatory proteins, possess the abiltiy to act on C3b attached to the same cell (intrinsic activity), the relative intrinsic versus extrinsic activity (for C3b bound to other cells or immune complexes) remains to be determined for MCP and HSV gC as well as for EBV.

Other herpes viruses have also been found to encode various proteins that interact with the humoral immune system. Thus, infection of human cells with other herpes viruses, including HSV-1, HSV-2, cytomegalovirus, and varicella zoster virus results in the appearance on the cell surface of virally encoded receptors for the Fc portion of human IgG (60-62). HSV-1 also induces the appearance of a C3b receptor in the plasma membrane of infected cells (46, 63), and this has been identified as a viral glycoprotein, gC (45, 47). This viral C3b receptor is reactive with an mAb to human CR1 (46); it also modulates complement activation as it has decay-accelerating activity for the alternative pathway C3 convertase, and interferes with the interaction of C3b with C5, although it lacks cofactor activity (45). Recently

gC of HSV-2 has been reported to possess the ability to bind iC3 (48). Other human pathogens including bacteria (64, 65) and fungi (66, 67) have been found to possess Fc or C3 binding properties. Although the biological significance of these various activities is far from clear, it is probable that they have evolved to provide survival value for the pathogens.

In this regard, the various EBV complement regulatory activities reported in this paper may enable EBV and EBV-infected cells to evade destruction by humoral immune mechanisms. Since EBV and EBV-infected cells activate complement, the virions as well as the infected cells would become coated with C3b as well as other complement components on contact with plasma. This could facilitate cytolytic destruction of the virus or virus-infected cell by either complement- or lymphocyte-dependent cytolytic mechanisms (68); it could also augment opsonization and phagocytic destruction by complement receptor–bearing cells (23). Furthermore, the ability of the virus to accelerate the decay of the alternative pathway C3 convertase would retard additional activation, while the cofactor activity for both factor I–mediated cleavages of C3 as well as C4 would rapidly convert bound C3b and C4b to bound C3dg and C4d which do not readily interact with phagocytic CR1 and CR3 receptors. The ability of EBV to facilitate the degradation of C3b on the viral surface to C3dg may restore infectivity by permitting the virus to again bind to CR2 via C3dg. These various activities could thus all be viewed as promoting viral survival. Since the effects on EBV infectivity can be experimentally determined, further study will indicate whether they do indeed permit EBV to evade destruction by complement- and cell-dependent host defense mechanisms.

## Summary

Serum incubated with purified EBV was found to contain C3 cleavage fragments characteristic of C3c. Since the cofactors necessary for such cleavage of C3b by factor I are not normally present in serum, EBV was tested for factor I cofactor activity. Purified EBV from both human and marmoset EBV-producing cell lines was found to act as a cofactor for the factor I–mediated breakdown C3b to iC3b and iC3b to C3c and C3dg. EBV also acted as a cofactor for the factor I–mediated cleavage of C4b to iC4b and iC4b to C4c and C4d. EBV from both the human and marmoset cell lines accelerated the decay of the alternative pathway C3 convertase. The classical pathway C3 convertase was unaffected. Multiple lines of evidence eliminated the possibility that marmoset or human CR1 was responsible for the functional activities of EBV preparations. The spectrum of activities was different from CR1 in that EBV and EBV-expressing cell lines failed to rosette with C3b or particles bearing C3b, the primary functional assay for CR1, and EBV did not accelerate classical pathway C3 convertase decay, another property of CR1. In addition, CR1 could not be detected immunologically on marmoset or human EBV-expressing cells and mAbs to CR1 failed to alter EBV-produced decay acceleration and factor I cofactor activities, although the antibodies blocked the same CR1-dependent functional activities. The multiple complement regulatory activities exhibited by purified EBV derived from human and marmoset cells differ from those of any of the known C3 or C4 regulatory proteins. These various activities would be anticipated to provide survival value for the virus by subverting complement- and cell-dependent host defense mechanisms.

## References

1. Henle, G., W. Henle, and V. Diehl. 1968. Relationship of Burkitt tumor associated herpes-type virus to infectious mononucleosis. *Proc. Natl. Acad. Sci. USA.* 59:94.

2. De-Thé, G., and Y. Zeng. 1982. Epidemiology of Epstein-Barr virus: recent results on endemic virus, non-endemic Burkitt's lymphoma and EBV related prenasopharyngeal carcinoma conditions. *In* Advances in Comparative Leukemia Research. D. S. Yohn and J. R. Blakeslee, editors. Elsevier Science Publishing Co., Inc., New York. 3–21.

3. Henle, W., V. Diehl, G. Kohn, H. Zür-Hausen, and G. Henle. 1967. Herpes-type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. *Science (Wash. DC).* 157:1064.

4. Purtillo, D. T., L. Hutt, J. Bhowen, J. P. S. Yang, C. Cassel, S. Allegra, and F. S. Rosen. 1978. Immunodeficiency to the Epstein-Barr virus in the X-linked recessive lymphoprolifer-ative syndrome. *Clin. Immunol. Immunopathol.* 9:147.

5. Alspaugh, M. A., G. Henle, E. T. Lennette, and W. Henle. 1981. Elevated levels of anti-bodies to Epstein-Barr virus antigens in sera and synovial fluids of patients with rheumatoid arthritis. *J. Clin. Invest.* 67:1134.

6. Ferrell, P. B., C. T. Aitcheson, G. R. Pearson, and E. M. Tan. 1981. Seroepidemiological study of relationships between Epstein-Barr virus and rheumatoid arthritis. *J. Clin. Invest.* 67:681.

7. Miller, G., T. Shope, H. Lisco, D. Stitt, and M. Lipman. 1972. Epstein-Barr virus: trans-formation, cytolytic changes and viral antigens in squirrel monkey and marmoset leuko-cytes. *Proc. Natl. Acad. Sci. USA.* 69:383.

8. Einhorn, L., M. Steinitz, E. Yefenof, I. Ernberg, T. Bakacs, and G. Klein. 1978. Epstein-Barr virus (EBV) receptors, complement receptors and EBV infectivity of different lym-phocyte fractions of human peripheral blood. II. Epstein Barr virus studies. *Cell. Im-munol.* 35:43.

9. Jondal, M., and G. Klein. 1973. Surface markers on B and T lymphocytes. II. Presence of Epstein-Barr receptors on B lymphocytes. *J. Exp. Med.* 138:1365.

10. Greaves, M. F., G. Brown, and A. B. Rickinson. 1975. Epstein-Barr virus binding sites on lymphocyte subpopulations and the origin of lymphoblasts in cultured lymphoid cell lines and in the blood of patients with infectious mononucleosis. *Clin. Immunol. Im-munopathol.* 3:514.

11. Nemerow, G. R., and N. R. Cooper. 1984. Early events in the infection of human B lymphocytes by Epstein-Barr virus: the internalization process. *Virology.* 132:186.

12. Sixbey, J. W., E. H. Vesterinen, J. G. Nedrud, N. Raab-Traub, L. A. Walton, and J. S. Pagano. 1983. Replication of Epstein-Barr virus in human epithelial cells infected *in vitro*. *Nature. (Lond.).* 306:480.

13. Shapiro, I. M., and D. J. Volsky. 1983. Infection of normal human epithial cells by Epstein-Barr virus. *Science (Wash. DC).* 219:1225.

14. Jondal, M., G. Klein, M. B. A. Oldstone, V. Bokisch, and E. Yefenof. 1976. Surface markers on human B and T lymphocytes. VIII. Association between complement and Epstein-Barr receptors on human lymphoid cells. *Scand. J. Immunol.* 5:401.

15. Fingeroth, J. D., J. J. Wells, T. F. Tedder, J. L. Strominger, P. A. Bird, and D. T. Fearon. 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc. Natl. Acad. Sci. USA.* 81:4510.

16. Nemerow, G. R., R. Wolfert, M. E. McNaughton, and N. R. Cooper. 1985. Identification and characterization of the EBV receptor on human B lymphocytes and its relationship to the C3d receptor (CR2). *J. Virol.* 55:347.

17. Frade, R., M. Barel, B. Ehlin-Henriksson, and G. Klein. 1985. Gp140, the C3d receptor of human B lymphocytes is also the Epstein-Barr virus receptor. *Proc. Natl. Acad. Sci. USA.* 82:1490.

18. Nemerow, G. R., C. Mold, V. Keivens-Schwend, V. Tollefson, and N. R. Cooper. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J. Virol.* 61:1416.

19. McConnell, I., G. Klein, T. F. Lint, and P. J. Lachmann. 1978. Activation of the alternative complement pathway by human B cell lymphoma lines is associated with Epstein-Barr virus transformation of the cells. *Eur. J. Immunol.* 8:453.

20. Mayes, J. T., R. D. Schreiber, and N. R. Cooper. 1984. Development and application of an enzyme-linked immunosorbent assay for the quantitation of alternative pathway activation in human serum. *J. Clin. Invest.* 73:160.

21. Mold, C., B. M. Bradt, G. R. Nemerow, and N. R. Cooper. 1988. Activation of the alternative complement pathway by the Epstein-Barr virus envelope glycoprotein, gp350. *J. Immunol.* 140:3867–3874.

22. Nemerow, G. R., F. C. Jensen, and N. R. Cooper. 1982. Neutralization of Epstein-Barr virus by nonimmune human serum. Role of cross-reacting antibody to Herpes simplex virus and complement. *J. Clin. Invest.* 70:1081.

23. Cooper, N. R., and G. R. Nemerow. 1986. Complement dependent mechanisms of virus neutralization. *In* Immunobiology of the Complement System. G. Ross, editor. Academic Press, Inc., New York. 139–162.

24. Ross, G. D., and M. E. Medof. 1985. Membrane complement receptors specific for bound fragments of C3. *Adv. Immunol.* 37:217.

25. Ross, G. P., and J. D. Lambris. 1982. Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils, and erythrocytes. *J. Exp. Med.* 155:96.

26. Medof, M. E., K. Iida, C. Mold, and V. Nussenzweig. 1982. Unique role of the complement receptor CR1 in the degradation of C3b associated with immune complexes. *J. Exp. Med.* 156:1739.

27. Ross, G. D., J. D. Lambris, J. A. Cain, and S. L. Newman. 1982. Generation of three different fragments of bound C3 with purified I or serum I. Requirements for factor H or CR1 cofactor activity. *J. Immunol.* 129:2051.

28. Lachmann, P. J., M. K. Pangburn, and R. G. Oldroyd. 1982. Breakdown of C3 after complement activation. Identification of a new fragment C3g, using monoclonal antibodies. *J. Exp. Med.* 156:205.

29. Iida, K., L. Nadler, and V. Nussenzweig. 1983. Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody. *J. Exp. Med.* 158:1021.

30. Frade, R., B. L. Myones, M. Barel, L. Krikorian, C. Charriaut, and G. D. Ross. 1985. Gp140, a C3b binding membrane component of lymphocytes, is the B cell C3dg/C3d receptor (CR2) and is distinct from the neutrophil C3dg receptor (CR4). *Eur. J. Immunol.* 15:1192.

31. Vik, D. P., and D. T. Fearon. 1987. Cellular distribution of complement receptor type 4 (CR4): expression on human platelets. *J. Immunol.* 138:254.

32. Nagasawa, S., C. Ichihara, and R. M. Stroud. 1980. Cleavage of C4b by C3b inactivator: Production of a nicked form of C4b, C4b', as an intermediate cleavage product of C4b by C3b inactivator. *J. Immunol.* 125:578.

33. Fuhita, T., I. Gigli, and V. Nussenzweig. 1978. Human C4 binding protein. *J. Exp. Med.* 148:1044.

34. Pangburn, M. K., R. D. Schreiber, and H. J. Müller-Eberhard. 1977. Human comple-

ment C3b inactivator: isolation, characterization and demonstration of an absolute requirement for the serum protein β1H for cleavage of C3b and C4b in solution. *J. Exp. Med.* 146:257.

35. Fujita, T., and V. Nussenzweig. 1979. The role of C4 binding protein and β1H in proteolysis of C4b and C3b. *J. Exp. Med.* 150:267.

36. Medof, M. E., and V. Nussenzweig. 1984. Control of the function of substrate-bound C4b-C3b by the complement receptor CR1. *J. Exp. Med.* 159:1669.

37. Mitomo, K., T. Fujita, and K. Iida. 1987. Functional and antigenic properties of complement receptor types 2, CR2. *J. Exp. Med.* 165:1424.

38. Seya, T., J. R. Turner, and J. P. Atkinson. 1986. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J. Exp. Med.* 163:837.

39. Weiler, J. M., M. R. Daha, K. F. Austen, and D. T. Fearon. 1976. Control of the amplification convertase of complement by the plasma protein β1H. *Proc. Natl. Acad. Sci. USA.* 73:3268.

40. Gigli, I., T. Fujita, and V. Nussenzweig. 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding proteins and C3b inactivator. *Proc. Natl. Acad. Sci. USA.* 76:6596.

41. Fearon, D. T. 1979. Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 76:5867.

42. Iida, K., and V. Nussenzweig. 1981. Complement receptor is an inhibitor of the complement cascade. *J. Exp. Med.* 153:1138.

43. Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J. Immunol.* 129:184.

44. Medof, M. E., T. Kinoshita, and V. Nussenzweig. 1984. Inhibition of complement activation on the surface of cells after incorporation of decay accelerating factor (DAF) into their membranes. *J. Exp. Med.* 160:1558.

45. Fries, L. F., H. M. Friedman, G. H. Cohen, R. J. Eisenberg, C. H. Hammer, and M. M. Frank. 1986. Glycoprotein C of herpes simplex virus 1 is an inhibitor of the complement cascade. *J. Immunol.* 137:1636.

46. Kubota, Y., T. A. Gaither, J. Cason, J. J. O'Shea, and T. J. Lawley. 1987. Characterization of the C3 receptor induced by herpes simplex type 1 infection of human epidermal, endothelial, and A431 cells. *J. Immunol.* 138:1137.

47. Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines. 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature (Lond.).* 309:633.

48. McNearney, T. A., C. Odell, V. M. Holers, P. G. Spear, and J. P. Atkinson. 1987. Herpes simplex virus glycoproteins gC-1 and gC-2 bind to the third component of complement and provide protection against complement-mediated neutralization of viral infectivity. *J. Exp. Med.* 166:1525-1535.

49. Nemerow, G. R., and N. R. Cooper. 1981. Isolation of Epstein-Barr virus and studies of its neutralization by human IgG and complement. *J. Immunol.* 127:272.

50. Stinski, M. F. 1976. Human cytomegalovirus: glycoproteins associated with virions and dense bodies. *J. Virol.* 19:594.

51. Wentworth, B. B., and L. French. 1970. Plaque assay of cytomegalovirus strains of human origin. *Proc. Soc. Exp. Biol. Med.* 135:253.

52. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large-scale isolation of functionally active components of the human complement system. *J. Biol. Chem.* 256:3995.

53. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

54. Nemerow, G. R., M. F. E. Siaw, and N. R. Cooper. 1986. Purification of the Epstein-Barr virus C3d complement receptor of human B lymphocytes: antigenic and functional properties of the purified protein. *J. Virol.* 58:709.

55. Nelson, D. S. 1963. Immune adherence. *Adv. Immunol.* 3:131.

56. Teilland, J. L., C. G. Zalc, M. Fontaine, C. Braun, and G. Mahouy. 1982. Involvement of human membrane-associated complement components in the rosette formation between marmoset red blood cells and human leukocytes. *Cell. Immunol.* 66:254.

57. Wilson, J. G., W. W. Wong, P. H. Schur, and D. T. Fearon. 1982. Mode of inheritance of decreased C3b receptors on erythrocytes of patients with systemic lupus erythematosus. *N. Engl. J. Med.* 307:981.

58. Seya, T., V. M. Holers, and J. P. Atkinson. 1985. Purification and functional analysis of the polymorphic variants of the C3b/C4b receptor (CR1) and comparison with H, C4b binding protein (C4bp), and decay accelerating factor (DAF). *J. Immunol.* 135:2661.

59. Kinoshita, T., M. E. Medof, K. Hong, and V. Nussenzweig. 1986. Membrane bound C4b interacts endogenously with complement receptor CR1 of human red cells. *J. Exp. Med.* 164:1377–1388.

60. Keller, R., R. Peitchel, J. N. Goldman, and M. Goldman. 1976. An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts. *J. Immunol.* 116:772.

61. Para, J. F., L. Goldstein, and P. G. Spear. 1982. Similarities and differences in the Fc binding glycoprotein (gE) of herpes simplex virus types 1 and 2 and tentative mapping of the viral gene for this glycoprotein. *J. Virol.* 41:137.

62. Ogata, M., and S. Shigeta. 1979. Appearance of immunoglobulin G Fc receptor in cultured human cells infected with varicella-zoster virus. *Infect. Immun.* 26:770.

63. Cines, D. B., A. P. Lyss, and M. Bina. 1982. Fc and C3 receptors induced by herpes simplex virus on cultured human endothelial cells. *J. Clin. Invest.* 69:123.

64. Dossett, J. H., G. Kronvall, R. C. Williams, Jr., and P. G. Quie. 1969. Antiphagocytic effects of Staphylococcal protein A. *J. Immunol.* 103:1405.

65. Burova, L. A., P. Christensen, R. Grubb, A. Jonsson, G. Samuelsson, C. Schalen, and M. L. Svenson. 1980. Changes in virulence, M protein and IgG Fc receptor activity in a type 12 group A streptococcal strain during mouse passages. *Acta Pathol. Microbiol. Scand. Sect. B Microbiol.* 88:199.

66. Heidenreich, F., and M. P. Dierich. 1985. *Candida albicans* and *Candida stellatoidea*, in contrast to other *Candida* species, bind iC3b and C3d but not C3b. *Infect. Immun.* 50:598.

67. Edwards, J. E., Jr., T. A. Gaither, J. J. O'Shea, D. Rotrosen, T. J. Lawley, S. A. Wright, M. M. Frank, and I. Green. 1986. Expression of specific binding sites on Candida with function and antigenic characteristics of human complement receptors. *J. Immunol.* 137:3577.

68. Ramos, O. F., G. Sarmay, E. Klein, E. Yefenof, and J. Gergely. 1985. Complement-dependent cellular cytotoxicity: lymphoblastoid lines that activate complement component 3 (C3) and express C3 receptors have increased sensitivity to lymphocyte-mediated lysis in the presence of fresh human serum. *Proc. Natl. Acad. Sci. USA.* 82:5470.