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Epstein–Barr virus (EBV) infection of murine L cells expressing recombinant human EBV/C3d receptor

(complement receptor type 2/Epstein–Barr nuclear antigen/L cell/latency)

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ABSTRACT **The normal host range of Epstein–Barr virus (EBV) is limited to primate B lymphocytes and certain epithelial cells that express the C3d/EBV receptor [complement receptor 2 (CR2, CD21)]. In the present study, expansion of the tissue tropism of EBV has been accomplished by stably transfecting the murine fibroblast L cell line with pMT.CR2. neo.1, a eukaryotic expression vector promoting the transcription of a complementary DNA insert encoding human CR2. High CR2-expressing transfected L cells were selected by fluorescence-activated cell sorting. The recombinant CR2 was shown to have the same molecular weight as wild-type CR2 from Raji cells and to mediate the binding by the transfectants of particles bearing the iC3b and C3d fragments of the third component of complement. All CR2-expressing L cells, but not nontransfected controls, also bound EBV, as assessed by indirect immunofluorescence. After a 60-hr culture, ≈0.5% of the CR2-expressing cells preincubated with EBV demonstrated immunofluorescent staining of EBV nuclear antigen with serum from a patient with nasopharyngeal carcinoma. No fluorescent staining of cells was seen with monoclonal antibodies to the early antigen complex or to gp350/220, indicating that the infection was predominantly latent. Infected cells cultured for up to 4 weeks remained EBV nuclear antigen-positive. The capacity of recombinant human CR2 to confer on murine L cells susceptibility to stable latent infection by EBV indicates that this receptor is a primary determinant of the tissue tropism of EBV and may facilitate studies of cell-specific factors that regulate the viral growth cycle.**

A relationship between cellular receptors for the third component of complement and for the Epstein–Barr virus (EBV), a human herpesvirus associated with infectious mononucleosis, Burkitt lymphoma, and nasopharyngeal carcinoma, was first suggested by Jondal *et al.* (1), who noted that the class of lymphocyte infected by EBV, the B lymphocyte, corresponded to the subset expressing C3 receptors. The molecular identification of two C3 receptors on B cells, complement receptor type 1 (CR1, CD35) (2) and type 2 (CR2, CD21) (3, 4), made possible subsequent experiments demonstrating that monoclonal antibody (mAb) to the latter, but not the former, receptor blocked binding of EBV to B lymphoblastoid cells and inhibited EBV-induced B-cell proliferation and differentiation (5, 6). In addition, purified CR2 was shown to bind EBV, and purified or recombinant gp350/220, the major envelope protein of EBV, specifically adsorbed CR2 from a detergent lysate of B-cell membrane proteins (5, 7, 8). This interaction may be mediated by a region in gp350/220 that is homologous to a sequence in the C3dg fragment of C3 (7, 8).

Although CR2 is generally considered to be expressed primarily by B cells, it may be responsible for the capacity of EBV to infect other cell types. Certain epithelial cells commonly (9, 10) and T lymphoma cells rarely (11) have been shown to be infected by the virus, and undifferentiated epithelial cells and these unusual T lymphomas express an antigen that is recognized by monoclonal anti-CR2 (11, 12). However the correlation between the presence of CR2 and susceptibility to infection by EBV is not always evident. For example, the MOLT-4 T-cell line expresses CR2 and is capable of binding EBV (5) but apparently cannot be infected by the virus. Follicular dendritic cells of the spleen also express CR2 antigen (13) but have not been reported to be infected *in vivo*. Although transient infection with EBV of CR2-nonexpressing cells has been achieved with the transfer of receptor from CR2-positive cells through membrane fusion (14, 15), long-term stable infection has not been reported. Thus, other tissue-specific factors may have a role in determining the tissue tropism of EBV. Further support for the existence of critical species- and/or cell type-specific factors was the demonstration that an EBV replicon comprised of *ori-P* and *EBNA-1* replicated in cells of various developmental lineages and several species but not in rodent fibroblasts (16).

With the molecular cloning of CR2 cDNA (17, 18), it has become possible to assess more directly the relative role of the receptor in the infection of cells with EBV. In the present study, murine L cells have been stably transfected with a vector promoting the transcription of an insert encoding CR2, and these cells have been compared to wild-type L cells for their capacity to bind EBV and to sustain infection by this virus.

MATERIALS AND METHODS

Antibodies. HB5 and OKB7 (Ortho) are IgG2a mouse monoclonal antibodies (mAbs) that specifically recognize human CR2 (4, 7). UPC10 is an IgG2a mouse mAb that recognizes levan and inulin (Bionetics, Kensington, MD). The mAb R3.3 is an IgG1 that recognizes a component of the EBV early antigen–D complex (19), which is encoded by the cell line B95-8 BMRF1 open reading frame (20), and 72A1 is an IgG mAb that recognizes the EBV gp350/220 glycoprotein (21). Fluorescein conjugated goat anti-human C3 (Organon Teknika–Cappel, Malvern, PA) and fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) were purchased. Nasopharyngeal carcinoma (NPC) patient serum 00448 was obtained from S. Spring (National Cancer Institute, Bethesda, MD).

Cell Lines. The EBV-positive Burkitt lymphoma cell line Raji and the EBV-transformed marmoset leukocyte cell line

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Abbreviations: EBV, Epstein–Barr virus; CR2, complement receptor type 2, which is the human C3d/EBV receptor; NPC, nasopharyngeal carcinoma; FBS, fetal bovine serum; BSA, bovine serum albumin; PBSA, phosphate-buffered saline containing 0.1% bovine serum albumin; mAb, monoclonal antibody; SCR, short consensus repeat.

B95-8 (22) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. The L-M (TK^-) cell line (23) was cultured in Dulbecco's modified Eagle's medium with D-glucose at 4500 mg/liter supplemented with 10% FBS and 2 mM glutamine or in medium containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine, and 3 μM glycine (HAT medium).

Expression of the CR2 cDNA. Clone λ4.11 was isolated from a human tonsillar cDNA library in λgt11 (18, 24). A 160-base-pair (bp) *Ssp* I/*Eco*RI fragment from the 5′ end of the λ4.11 cDNA insert was used as a probe to detect the cDNA clone, λ1.3, which extended to the 5′-untranslated region.

The cDNA inserts in λ1.3 and λ4.11, which overlapped at a unique *Nsi* I site, were removed by *Eco*RI digestion and subcloned into pUC18 and pBR322, respectively. The λ1.3 insert was isolated with a *Sal* I/*Nsi* I digest, the λ4.11 insert was isolated with an *Nsi* I/*Nhe* I digest, and the pMT.neo.1 vector (provided by K. Peden, John Hopkins University) was restricted with *Xho* I and *Xba* I. The three fragments were mixed at equimolar ratios, ligated, and transfected into *Escherichia coli* DH5 α cells. A recombinant clone, pMT.CR2.neo.1 (Fig. 1), was found by restriction mapping to contain the full-length CR2 cDNA in the desired orientation.

Mouse L-M (TK^-) cells were transfected with a calcium phosphate precipitate of pMT.CR2.neo.1 (25). Colonies resistant to G418 were pooled and assayed for expression of CR2 by indirect immunofluorescence by incubation of cells at 0°C with mAb UPC10 at 1.0 μg/ml or mAb HB5 at 1.0 μg/ml, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 10 μg/ml.

Flow cytometric analysis was performed with a Coulter Epics model 752 counter. Autofluorescence was corrected as described (26). One million transformants stained with mAb HB5 were sorted with recovery of the brightest 5%, which were cultured for 2 weeks. One million of these cells were sorted with recovery of the brightest 12%, which were cultured and used in the remainder of the experiments.

L cells expressing CR2 (3.5×10^7) or 4.3×10^7 Raji cells were surface labeled by use of Iodo-Gen (4) (Pierce). Detergent lysates of labeled cells were sequentially immunoadsorbed with Sepharose-UPC-10 and Sepharose-HB5, and adsorbed proteins were eluted by boiling in 1% $NaDodSO_4$ and subjected to electrophoresis on $NaDodSO_4$/5% polyacrylamide gels.

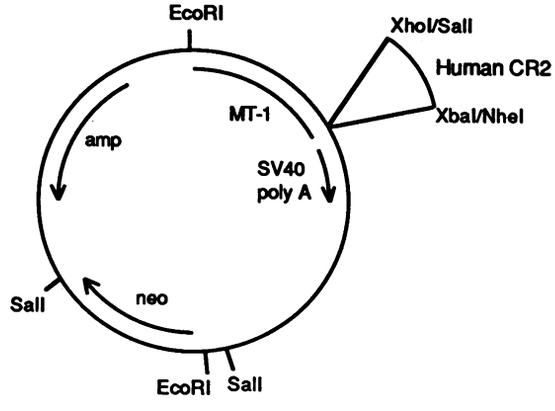

FIG. 1. Expression construct pMT.CR2.neo.1. Full-length human CR2 cDNA was cloned into the *Xho* I/*Xba* I sites of pMT.neo.1, where expression is controlled by the mouse metallothionein I promoter, and splice and polyadenylylation signals are from the simian virus 40 early region. The vector contains a marker gene, *neo*, which codes for the aminoglycoside 3′-phosphotransferase from transposon Tn5 and confers resistance to the antibiotic G418. An origin of replication and an ampicillin-resistance gene enable propagation and selection in *E. coli*.

Rosette Assays with Erythrocyte Intermediates Bearing Fragments of C3b, iC3b, and C3d. Erythrocytes bearing C3b were prepared by sequential incubation of 10^9 EAC4 (Diamedix, Miami) with 2000 units of C1, 2000 units of C2, and 400 μg of C3. EAC1,4,2,3b was converted to EAC4,3b by incubation for 60 min at 37°C in gelatin/veronal/saline buffer (GVS) containing 10 mM EDTA. The EAC4,3b was converted to EAC4,i3b by incubation for 30 min at 37°C in GVS containing 40 mM EDTA and 5% normal human serum. The EAC4,i3b was converted to EAC4,3d by incubation with trypsin at 5 μg/ml for 10 min at 37°C (4).

Replicate samples of 12.5×10^6 EAC4,3b; EAC4,i3b; or EAC4,3d were incubated with 10^6 Raji cells, nontransfected L cells, or CR2-bearing L cells at room temperature for 60 min in 0.1 ml of Hanks' balanced salt solution containing 0.1% bovine serum albumin (BSA). For blocking studies, the Raji cells or L cells were preincubated for 30 min at 4°C with mAb UPC10, HB5, or OKB7 all at 5 μg/ml before addition of EAC4,3d. Rosette formation was assessed by examining 200 nucleated cells in wet-mount preparations by light microscopy, with cells having >4 erythrocytes attached being scored as positive rosettes.

Assay for Cellular Binding and Infection by EBV. The B95-8 strain of EBV was prepared as described (27). Raji cells (10^6) or L cells (10^6) in 0.05 ml of phosphate-buffered saline containing 0.1% BSA (PBSA) were incubated with an equal volume of purified EBV for 60 min at 0°C and washed three times with PBSA. Half of each cell preparation was incubated at 0°C for 30 min in PBSA containing a 1:50 dilution of 72A1 anti-gp350 mAb hybridoma culture supernatant, washed, and incubated with FITC-conjugated goat anti-mouse IgG at 10 μg/ml at 0°C for 30 min, after which the cells were assessed microscopically for fluorescence. The other half of each cell preparation was divided into replicate samples of 5×10^4 cells that were cultured for 60 hr on chamber slides. Some slides were air-dried and fixed in acetone or methanol at −20°C. Cells on duplicate slides were split 1:6 and cultured for an additional 1, 2, or 4 days, after which they were air dried and fixed in either acetone or methanol. The methanol-fixed slides were incubated with a 1:50 dilution of NPC patient serum 00448 and 10% fresh human serum at 37°C for 30 min. The slides were washed with PBSA and incubated with FITC-conjugated goat anti-human C3 at 10 μg/ml at 37°C for 30 min. Slides that had been acetone-fixed were incubated at 37°C for 30 min with either a 1:5000 dilution of R3.3 or a 1:50 dilution of 72A1 hybridoma culture supernatant, washed with PBSA, and incubated at 37°C for 30 min with FITC-conjugated goat anti-mouse IgG at 10 μg/ml.

RESULTS

Expression of Recombinant CR2 by Murine L Cells. The L-M (TK^-) cells were transfected with pMT.CR2.neo.1 and selected for 2 weeks in medium containing G418. The selected cells, but not nontransfected, wild-type L cells, were indirectly stained with anti-CR2 mAb HB5. The fluorescence intensity among the transfectants varied widely, ranging from none to greater than that of the positive control, the Raji Burkitt lymphoma cell line. Therefore, transfectants expressing relatively large amounts of CR2 were selected by two rounds of fluorescence-activated cell sorting after indirect staining with mAb HB5. After culture, the positively sorted cells were shown to express equivalent or greater amounts of CR2 when qualitatively compared to Raji cells for fluorescence intensity after immunofluorescent labeling with mAb HB5 (data not shown).

The membrane protein on the transfected L cells reactive with the anti-CR2 mAb was assessed by immunoprecipitation of ^{125}I-surface-labeled cells and $NaDodSO_4$/PAGE. The

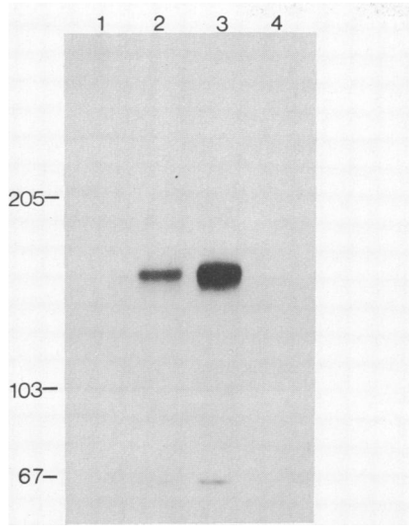

FIG. 2. Expression of recombinant human CR2 as assessed by immunoprecipitation. Autoradiograph of $NaDodSO_4$/5% PAGE of ^{125}I-labeled membrane proteins that had been immunoprecipitated with HB5 mAb from Raji cells (lane 2) and from L cells transfected with pMT.CR2.neo.1 (lane 3) and with mAb UPC10 from Raji cells (lane 1) and from the transfected L cells (lane 4).

recombinant CR2 comigrated with CR2 from Raji cells with an M_r of 145,000 (Fig. 2).

The cDNA clone, λ4.11, used for construction of pMT.CR2.neo.1 encodes a form of CR2 having 15 short consensus repeats (SCRs) (18). However, cDNA clones specifying a 16-SCR form of CR2 also have been identified (17, 18). To determine whether the 15-SCR form of CR2 expressed on L cells was capable of binding the appropriate C3 fragments, erythrocyte intermediates bearing C3b, iC3b, and C3d, respectively, were prepared and assayed for binding to L cells expressing recombinant CR2. As was observed with the Raji cells, the CR2-expressing L cells preferentially formed rosettes with erythrocytes bearing the iC3b and C3d fragments (Fig. 3 and Table 1). In addition, these rosettes

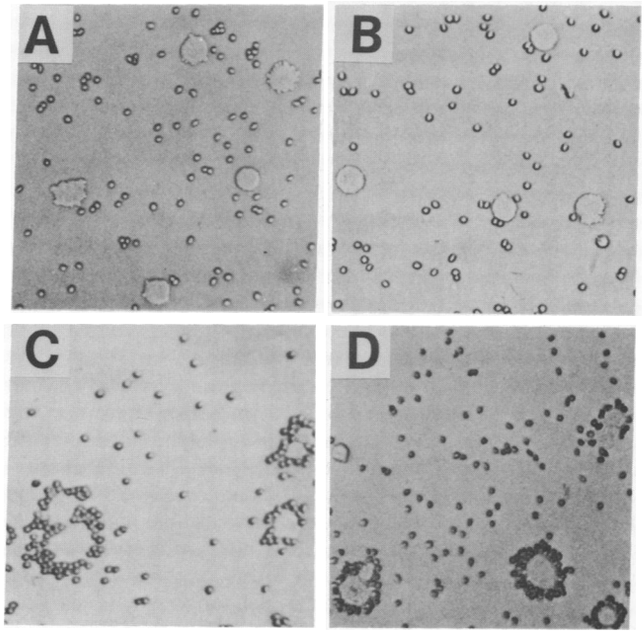

FIG. 3. Rosette formation between pMT.CR2.neo.1-transfected L cells and the erythrocyte intermediates EAC4 (*A*), EAC4,3b (*B*), EAC4,i3b (*C*), and EAC4,3d (*D*). (×30.)

Table 1. Formation of C3-dependent rosettes with L cells expressing recombinant CR2

	Cells forming rosettes with erythrocyte intermediates, %				
			EAC4,3d mAb preincubation		
Cell type	EAC4,3b	EAC4,i3b	None	HB5	OKB7
L	0	0	0	—	—
CR2-L	0	58	66	70	0
Raji	11	93	93	97	8

Two hundred cells in each assay were assessed for rosette formation; >4 erythrocytes bound per nucleated cell was considered as positive.

were inhibited by the anti-CR2 mAb OKB7 but not by mAb HB5 in accordance with previous studies of the effects of these mAbs on CR2 function (4, 6, 28) (Table 1).

EBV Binding to and Infection of L Cells Expressing Recombinant CR2. Wild-type L cells, L cells expressing CR2, and Raji cells were incubated with purified B95-8 strain of EBV, washed, and assessed for cell-bound EBV by indirect immunofluorescence with anti-gp350/220 mAb. Fluorescent speckles were observed on the surface of the CR2-expressing L cells but not on the wild-type L cells incubated with EBV (Fig. 4). This pattern of fluorescence on the L cells was identical to, though greater in magnitude than, that present on Raji cells that had been incubated with EBV (Fig. 4).

Replicate samples of the wild-type and CR2-expressing L cells that had been preincubated with EBV were cultured for 60 hr and assayed for EBV-encoded proteins by indirect immunofluorescence. Approximately 0.5% of the CR2-expressing L cells that had been incubated with EBV demonstrated bright granular nuclear staining with serum from a patient with NPC. This granular pattern of nuclear immunofluorescence typical of EBV nuclear antigen was similar to that observed in latently infected Raji cells (data not shown). Immunofluorescent staining of the infected, CR2-expressing L cells with mAbs directed to the EBV early antigen–D complex and gp350/220 was negative. No staining with NPC patient serum was seen with wild-type L cells preincubated with EBV, or with CR2-expressing L cells in the absence of EBV.

Maintenance of EBV in CR2-expressing L cells was assessed by splitting the cells 1:6 and culturing replicate samples for 1, 2, and 4 days, respectively, after which cells were stained indirectly with NPC patient serum. Although the number of foci containing cells having nuclei staining with the antiserum remained constant, the number of cells in each focus appeared to double each 24 hr, going from one to approximately eight per cluster (Fig. 5). No evidence of cell lysis was observed at any time.

To verify that the infected cells were mouse fibroblasts, the cultures were propagated in HAT medium. After one week, all cells had died, consistent with the TK^- phenotype of the L cells.

DISCUSSION

In the present study, a murine fibroblast cell line has been rendered susceptible to persistent infection by EBV through the stable expression of a single human membrane protein, CR2. This finding suggests that the host range of this virus is limited primarily by primate CR2, with other tissue- and species-specific factors being less important and raises the possibility that many cell types not normally infected by EBV may be converted to this state by inducing expression of this receptor.

The three components of this model system addressing the question of the relative role of CR2 in determining the tissue

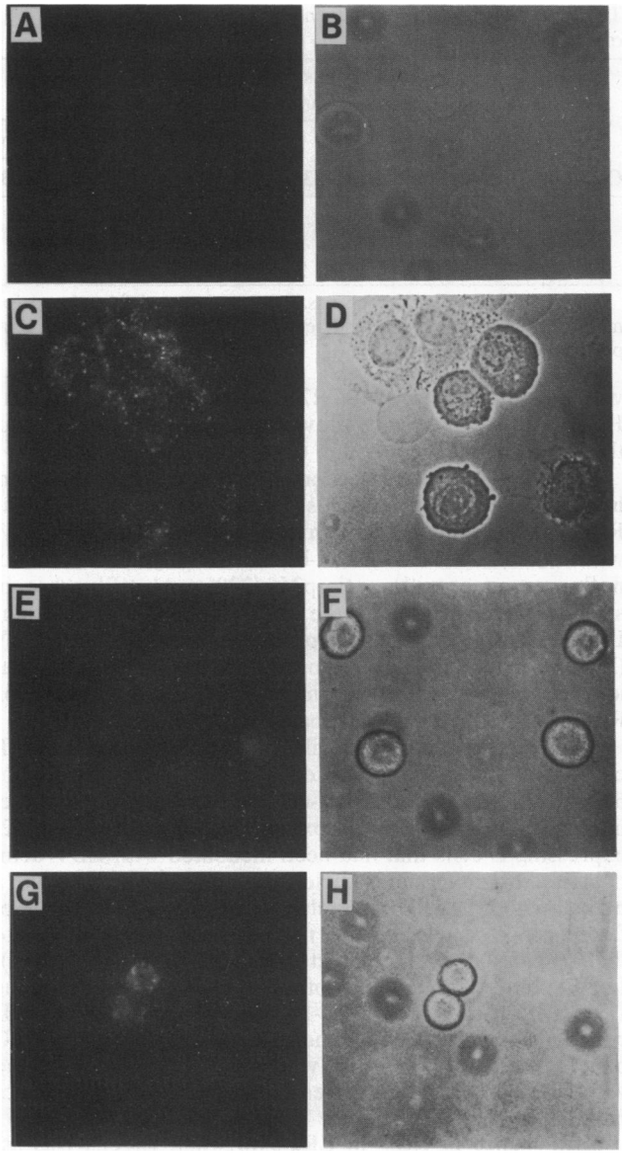

FIG. 4. Binding of EBV to L cells expressing human CR2. Cells were incubated with or without EBV and stained by indirect immunofluorescence with 72A1, an anti-gp350/220 mAb. Wild-type L cells incubated with EBV (*A* and *B*), CR2-L cells incubated with EBV (*C* and *D*), Raji cells incubated without EBV (*E* and *F*), and Raji cells incubated with EBV (*G* and *H*) were photographed with either fluorescence (*A*, *C*, *E*, and *G*) or phase-contrast (*B*, *D*, *F*, and *H*) microscopy (×95).

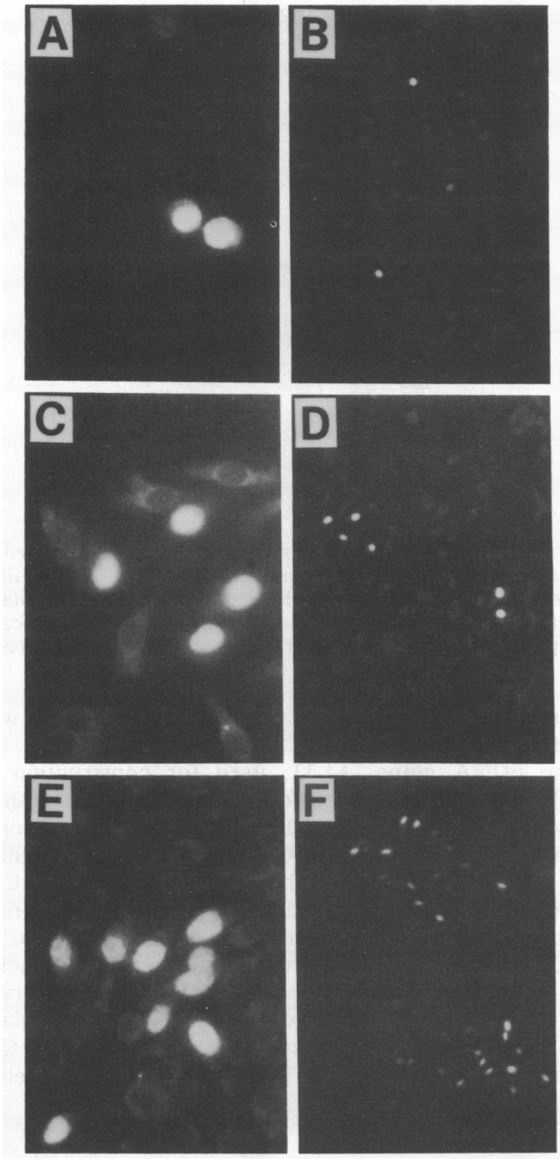

FIG. 5. Time-course of EBV-infected L cells in culture. EBV-infected L cells were grown to confluence, split 1:6, and stained by indirect immunofluorescence with NPC patient serum. Fluorescence photomicrographs were taken of representative fields at original magnification of ×95 (*A*, *C*, and *E*) and ×10 (*B*, *D*, and *F*) after 24 hr (*A* and *B*), 48 hr (*C* and *D*), and 96 hr (*E* and *F*).

tropism of EBV were the 15-SCR form of CR2, the expression vector pMT.neo.1, and the murine L cell. The choice of each of these variables reflected specific concerns. The primary structure of CR2 has been derived from sequences of cDNA clones from tonsillar (18) and Raji lymphoblastoid cell (17) libraries. The entire extracellular domain is comprised of tandemly repeated SCRs of 60–70 amino acids having four invariant cysteines that engage in disulfide bonding that may lead to formation of a triple-loop structure. There is a single hydrophobic putative transmembrane region and a COOH-terminal cytoplasmic tail of 34 amino acids. The SCR, one or several of which must constitute the binding sites for both C3d and EBV, also is the basic structural motif of the other C3/C4 binding proteins encoded by linked genes at 1q32 (29, 30).

Although analyses of the 145,000 M_r CR2 glycoprotein (3, 4, 7), including biosynthetic studies (31), had recognized only a single form of the receptor, two types of CR2 cDNA clones have been found, which differ by the number of SCRs they encode: the more frequently observed type of clone in the tonsillar cDNA library specified 15 SCRs, whereas the second type, which was also observed in the Raji cell library, specified an additional SCR of 59 amino acids between SCR-10 and -11 of the 15-SCR form (18). Therefore, by expressing the 15-SCR form in L cells and demonstrating that it comigrated on $NaDodSO_4$/PAGE with CR2 immunoprecipitated from Raji cells (Fig. 2) and had iC3b-, C3d-, and EBV-binding functions (Figs. 3 and 4; Table 1), we offer evidence that the additional SCR is not required. However, we have not compared the two forms of the receptor and cannot exclude some secondary enhancing role for the additional SCR in these functions.

The expression vector pMT.neo.1 was chosen for these studies because the mouse metallothionein I promoter (32) apparently offered the potential for upregulating CR2 expres-

sion through addition of heavy metal cations. Although EBV-negative Burkitt lymphomas expressing low numbers of CR2 can be infected with EBV (33, 34), we considered the possibility that non-B cells, such as the L cell, might require higher levels of CR2 expression for infection. For example, the human T cell line MOLT-4, which expresses low levels of CR2 and is capable of binding EBV (5), is not infected by simple coculture with the B95-8 strain. However, the L cells stably transfected with pMT.CR2.neo.1 constitutively expressed CR-2 and addition of cadmium increased the level of expression by only 2-fold (data not shown). Thus, fluorescence-activated cell sorting of transfectants stained with anti-CR2 was used to select for L cells having high constitutive levels of CR2 that were at least equivalent to those observed on Raji cells. This high level of receptor expression mediated the uptake of relatively large amounts of EBV (Fig. 4) and may have contributed to the capacity of these cells for infection by the virus (Fig. 5).

The L-M (TK^-) murine fibroblast cell line was considered to represent a stringent test of the hypothesis that human CR2 is the primary determinant of the tissue tropism of EBV. Not only is this cell type not of lymphocytic lineage or of a primate species, but previous studies with an EBV replicon plasmid containing *ori-P* and *EBNA-1* suggested that rodent cells could not sustain replication of the EBV episome (16). The CR2-expressing L cells that were infected with EBV continued to express EBV-encoded nuclear antigens for up to 4 weeks, and the number of cells expressing viral antigens within a focus doubled with a time course comparable with the doubling time of the fibroblasts (Fig. 5), indicating that the EBV genome is maintained in these cells. However, it has not yet been determined whether the viral DNA exists as an episome or is integrated into the genome of the L cells.

The EBV-infected, CR2-transfected L cells expressed nuclear antigens detected by the NPC serum, but no antigens detectable by mAbs directed to the lytic cycle proteins, gp350/220 and early antigen. Although the number of infected cells examined during the course of these studies was only in the range of several hundred, this observation indicates that the infection was predominantly latent. Our finding contrasts with studies of the infection of human epithelial cells *in vitro* in which EBV-encoded antigens of the lytic cycle were expressed (35) and is more reminiscent of viral infection of human B cells. The capacity to infect a variety of different cell types through expression of recombinant human CR2 should facilitate the analysis of those cellular factors that influence the viral growth cycle and the mechanism by which latent infection may induce cellular immortalization.

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