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Replication of a Macrophage-Tropic Strain of Human Immunodeficiency Virus Type 1 (HIV-1) in a Hybrid Cell Line, CEMx174, Suggests that Cellular Accessory Molecules Are Required for HIV-1 Entry

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To investigate the mechanism underlying one aspect of the cellular tropism of human immunodeficiency virus type 1 (HIV-1), we used a macrophage-tropic isolate, 89.6, and screened its ability to infect a number of continuous cell lines. HIV-1(89.6) was able to replicate robustly in a T-cell/B-cell hybrid line, CEMx174, while it replicated modestly or not at all in either of its parents, one of which is the CD4-positive line CEM.3. Analysis by transfection of a molecular clone, a virus uptake assay, and polymerase chain reaction all provided strong evidence that the block to HIV-1(89.6) replication in the CEM.3 line lies at the level of cellular entry. These results were complemented by preparing a CD4-expressing derivative of the B-cell parent, 721.174, and demonstrating that it is permissive for productive HIV-1(89.6) replication. Given these experimental findings, we speculate that there exist cellular accessory factors which facilitate virus entry and infection in CD4-positive cells. Furthermore, these cellular accessory factors may be quite virus strain specific, since not all macrophage-tropic strains of HIV-1 were able to replicate in the CEMx174 hybrid cell line. This experimental model provides a system for the identification of one or more of these putative cellular accessory factors.

Isolates of human immunodeficiency virus type 1 (HIV-1) differ significantly in cellular host range and are frequently classified by the ability to productively infect primary cells in vitro (6, 8, 9, 17, 18). Lymphocyte-tropic (L-tropic) isolates can replicate in peripheral blood lymphocytes (PBLs) but not in monocyte-derived macrophages (MDM), whereas isolates that are classified as macrophage tropic (M tropic) replicate well in both PBLs and MDM (6, 9, 19, 24, 36). Many L-tropic isolates yield highly cytopathic infections in PBLs, inducing cell killing and syncytium formation, while infection of either MDM or PBLs with M-tropic isolates results in more modest cytopathology. In addition, many L-tropic isolates replicate in continuous cell lines, whereas most M-tropic strains fail to do so as effectively.

Several recent studies have focused on the viral elements which determine host cell tropism, using recombinant viruses generated between molecularly cloned L-tropic and M-tropic isolates. These experiments have mapped M tropism to regions of the envelope gene (*env*), particularly those encompassing the gp120 principal neutralizing domain (PND or V3 loop) (2, 3, 36, 48). The involvement of *env* has strongly suggested that the differences in tropism between L-tropic and M-tropic viruses lie at the level of viral entry. However, the cellular factors involved in differential host cell tropism are not understood. Since CD4 is clearly the major receptor for both PBLs and MDM (7, 9, 42) and since the CD4 binding domain does not appear to be a determinant of M tropism, several investigators have speculated that there are accessory molecules that are important for viral penetration into macrophages. These molecules would presumably be targeted by regions of *env*, including the PND, outside the CD4 binding region of gp120. Remarkably, tropism for immortalized cell lines and sensitivity to neutralization by recombinant soluble CD4 (rsCD4), both characteristics of L-tropic isolates, have also been mapped to the PND (27).

HIV-1(89.6) is an M-tropic isolate recovered from the peripheral blood of a severely immunocompromised individual that has recently been molecularly cloned (6). HIV-1(89.6) replicates to high titer in both MDM and PBLs but, unlike other M-tropic isolates, produces a highly cytopathic infection, resulting in syncytium formation in both cell types.

To further define the cellular host range of HIV-1(89.6), we attempted infection of a series of immortalized cell lines; representative results are summarized in Table 1. As with most M-tropic strains, HIV-1(89.6) did not replicate well in most T-cell lines, such as SupT1 or Molt 4 clone 8, or in the monocytoid cell line U937. However, HIV-1(89.6) replicated to high levels in a T-cell/B-cell hybrid line, CEMx174 (40). While HIV-1(89.6) produced an aggressive and cytopathic infection in CEMx174 cells, it was unable to replicate efficiently in either of the parental cell lines, including the CD4-positive parental T cell line, CEM.3.

Because of this paradox, we designed experiments to identify the level of restriction for HIV-1(89.6) in the CEM.3 cell line. Results from these experiments revealed that although HIV-1(89.6) infection in the hybrid cell line is CD4 dependent, at least one block to infection of CEM.3 cells was at the level of viral entry, suggesting that CD4 alone is not sufficient for viral penetration. This experimental paradigm will provide a model for future studies to determine those cellular factors that, in addition to CD4, are involved in viral entry of this M-tropic isolate.

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TABLE 1. Infection of CEM.3, CEMx174, 721.174, and other reference cell lines by L-tropic and M-tropic strains of HIV-1

Cells	p24 antigen production[a]	
	IIIB (L tropic)	89.6 (M tropic)
PBLs	++++	++++
Macrophages	+/−	+++
SupT1	++++	−
U937	++++	−
HeLa	−	−
HeLaT4	++++	+/−
CEMx174	++++	++++
CEM.3	++++	+/−
721.174	−	−

[a] Cells were infected with cell-free virus, and the level of replication was measured by p24 antigen production in the extracellular supernatant. ++++, productive infection (peak production of p24 of >500 ng/ml); +++, productive infection (peak production of p24 of 50 to 500 ng/ml); +/−, nonproductive infection ($p24^{gag}$ level of <5 ng/ml); −, no detectable infection.

MATERIALS AND METHODS

Cells. Rhabdomyosarcoma (RD) cells, which are adherent and CD4 negative, were maintained in RPMI medium with 1% L-glutamine and 10% fetal calf serum (FCS) and were used for transfection of infectious proviral DNA. 721.174 is a CD4-negative B-lymphoblastoma cell line (12), and CEM.3 is a CD4-positive T-lymphoblastoma cell line (12, 14, 25). CEMx174 is a CD4-positive T-cell/B-cell hybrid line generated from the polyethylene glycol-mediated fusion of 721.174 and CEM.3 cells (40). All three are nonadherent cell lines, originally a gift from Peter Cresswell, and were maintained in RPMI medium with 1% L-glutamine and 10% FCS. The lymphoid cell lines were periodically stained and analyzed by flow cytometry to ensure that expression of surface markers had remained stable. Surface CD4 staining was approximately the same on the CEMx174 and CEM.3 cell lines (>95%).

Virus. Viral stocks of HIV-1(IIIB)(LAI), a prototype L-tropic virus (38), and HIV-1(89.6), an M-tropic virus (6), were prepared either from an infectious molecular clone (pHXB2D or p89.6) or from seed stocks. Proviral DNA was transfected into RD cells by the calcium phosphate method (5 Prime-3 Prime, West Chester, Pa.). After transfection, infectious virus was rescued by cocultivation with CEMx174 cells and then propagated in CEMx174 cell cultures for three to four passages (approximately 2 weeks). High-titer cell-free viral supernatants were collected, filtered, and stored in 1-ml aliquots at −80°C for future studies. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by inoculation of CEMx174 cells plated in 48-well plates (Costar), using 6 wells per 10-fold dilution. Endpoints were calculated on the basis of the presence of syncytium formation in over 25% of the cells in each well, 14 days after infection (39).

Infections. Equal numbers of cells (10^6/ml) were incubated with equivalent amounts of infectious virus (as calculated from the $TCID_{50}$) in a total volume of 1 ml for 1 to 2 h at 37°C. Following incubation, the viral inoculum was removed and the cells were extensively washed with phosphate-buffered saline (PBS) and treated with trypsin (GIBCO-BRL; trypsin-EDTA; 500 μg/ml) to remove any residual virus. Supernatants were periodically collected and assayed for the presence of viral $p24^{gag}$ antigen by enzyme-linked immunosorbent assay (ELISA) (Coulter Corp., Hialeah, Fla.) and examined visually for the presence of cytopathic effect. For experiments using rsCD4, the virus was incubated with 50 μg of rsCD4 (a gift from Ray Sweet, SmithKline Beecham, King of Prussia, Pa.) per ml for 1 h at room temperature prior to inoculation of the cells, and then 1 to 2 μg of rsCD4 per ml was maintained in the culture medium (11).

Immunofluorescence assay (IFA). Infected cells were cytospun onto glass slides, washed in PBS, fixed in ice-cold acid alcohol for 10 min, and then washed again in PBS. For detection of viral antigen, we used a combination of three monoclonal antibodies against HIV-1 at a 1:500 dilution: 25.4 against viral core protein $p24^{gag}$ (gift from J. McClure), and 110.1 and 9201 against the carboxy terminus of the viral envelope glycoprotein gp120 (13, 31) (obtained from the National Institutes of Health AIDS Research and Reference Reagent Program). As a negative control, a monoclonal antibody to an unrelated viral glycoprotein (generated in our laboratory) was also used at a 1:500 dilution. Following a blocking step (10% goat serum for 1 h at room temperature), the cells were incubated with the primary antibody cocktail for 1 h at room temperature in a humidification chamber and then washed in PBS. The secondary goat anti-mouse $F(ab')_2$ fluorescein isothiocyanate-conjugated antibody (obtained from Sigma) was diluted 1:75 in 10% goat serum with 0.05% Evans blue and incubated for 1 h at room temperature in a humidification chamber and then washed finally with PBS. Citifluor (obtained from Citifluor Ltd., Northampton Square, London, England) was added to preserve the fluorescence of the cells, and the slides were stored in the dark at 4°C. The slides were then examined and photographed with a Leitz Aristoplan microscope.

Proviral transfections. To compare replication efficiencies of proviral clones, cells were transfected by using DEAE-dextran (5 Prime-3 Prime). Briefly, 10^7 cells were suspended in 1 ml of serum-free OPTI-MEM medium along with 5 μg of proviral DNA in 400 μg of DEAE-dextran per ml. The cell-DNA mixture was incubated at 37°C for 1 h, with agitation every 15 min, washed in serum-free medium, and then incubated in 10 ml of RPMI medium containing 1% L-glutamine, 1% penicillin-streptomycin, and 10% FCS. Supernatants were collected at regular time points after transfection and assayed by ELISA for the presence of viral $p24^{gag}$ antigen. The efficiency of transfection in each cell line was assessed by parallel transfection of a plasmid, pCDNA3/CAT (Invitrogen, San Diego, Calif.), which expresses the chloramphenicol acetyltransferase (CAT) gene under the control of the immediate-early cytomegalovirus promoter (43), and assaying for CAT activity by using a liquid scintillation assay (35). Specific CAT activity was calculated as the counts per minute above the blank level per microgram of protein in the sample, using the Lowry assay (32).

PCR. Cells (10^6) were infected with virus stocks (multiplicity of infection [MOI] = 0.01) that had been treated with 50 μg of RNase-free DNase I (obtained from Worthington, Freehold, N.J.) per ml for 1 h at room temperature. Control cultures were infected with virus that had been heat inactivated by treatment at 56°C for 1 h. After a 2-h incubation at 37°C, the inoculum was removed, and the cells were washed and treated with trypsin (Sigma trypsin-EDTA; 500 μg/ml) to remove residual virus and then incubated for 16 h at 37°C. The cells were then washed and lysed in 100 μl of lysis buffer (0.1% Nonidet P-40 in 100 mM KCl–20 mM Tris-HCl [pH 8.4] with 500 μg of proteinase K per ml) for 2 h at 60°C. The proteinase K was inactivated by boiling the reaction mixture for 15 min. Newly synthesized HIV-1 DNA was then detected by using primers spanning the long terminal repeat (LTR) U3-R-U5 region: LTR-1 [nucleotides 138 to 159 of the HIV-1(89.6) sequence; 5′ ACAAGCTAGTACCAGTTGAGCC-3′] and LTR-2 (nucleotides 562 to 541; 5′-GCACACACTACTTG

AAGCACTC-3'). Each polymerase chain reaction (PCR) mixture contained 10 μl of the cell lysate (equivalent to $\approx 10^5$ cells), 200 μM each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 1 μM each primer, and *Taq* DNA polymerase (2.5 U/100μl; Perkin-Elmer, Norwalk, Conn.). The reaction mixture (100 μl) was overlaid with 50 μl of mineral oil and subjected to 30 cycles of amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 60 s) followed by a 5-min extension at 72°C in a Perkin-Elmer thermocycler. PCR products were then analyzed by gel electrophoresis and visualized by ethidium bromide staining. To determine the sensitivity of the PCR amplification, DNA from the HIV-1-infected ACH-2 line, which contains a single proviral copy (5, 15, 16), was amplified in parallel. Serial 10-fold dilutions of the ACH-2 cells were diluted into uninfected CEMx174 cells to a total concentration of 10^6 cells, and the DNA was extracted and treated as indicated for the infected cells. Primers PCO4 (54 to 73) and GH20 (−195 to −176) (obtained from Perkin-Elmer), which amplify a 268-bp portion of the β-globin gene (1, 30), were used as DNA controls and included in the same reaction. The specificity of the HIV-derived amplification products was confirmed by transferring the DNA to Zeta-probe (Bio-Rad) and probing with ^{32}P-labeled oligonucleotide 5'-TGCTTTTTGCCTGTACTGGG TCTCTCTGGTTAGAC-3', corresponding to nucleotides 435 to 470 of the HIV-1(89.6) LTR.

Virus internalization assay. The assay was adapted for lymphoid cell lines from previously published protocols (21, 22). Equal numbers of cells (2×10^6) were incubated with 500 ng of virus per ml (standardized by the amount of $p24^{gag}$ per milliliter) for 1 h at 37°C. Following incubation, the viral inoculum was removed and the cells were extensively washed with PBS and treated with trypsin (2.5 mg/ml for 4 min at room temperature) to remove any residual, noninternalized virus. Immediately thereafter, the cells were lysed in 1% Triton and assayed for the presence of viral $p24^{gag}$.

Stable expression of CD4 in 721.174 cells. To generate CD4-expressing 721.174 cells, we used a retroviral vector, SCX, containing the full-length CD4 gene and the neomycin gene for eukaryotic selection (a generous gift from Richard Morgan, National Institutes of Health, Bethesda, Md.). Cells were infected by adding viral supernatant containing SCX or a control vector, G1N, which contains only the neomycin gene, supplemented with 5 μg of protamine sulfate per ml, and incubated for 8 to 12 h at 37°C. Following infection, the retroviral supernatant was removed and the cells were maintained in RPMI medium supplemented with 1% L-glutamine and 10% FCS. Three days after infection, the cells were selected and maintained in RPMI medium containing 200 μg of Geneticin (G418; GIBCO BRL, Gaithersburg, Md.) per ml for 14 days. Surface CD4 expression was determined by flow cytometry analysis.

Flow cytometry staining and analysis. For detection of surface molecules, the following monoclonal antibodies were used: Leu3a (Beckton Dickinson) or OKT4a (Ortho Pharmaceuticals), both of which recognize CD4; W6/32, which recognizes a constant domain of major histocompatibility complex class I (obtained from Accurate Scientific); CX2.1, directed against HLA-DR (a gift from James Hoxie); Leu16, which recognizes an early B-cell marker (obtained from Becton Dickinson); and control supernatant from P3 myeloma cells. Cells were washed in staining buffer (SB; PBS containing 0.1% bovine serum albumin and 0.02% sodium azide) and incubated with the primary antibody for 30 min on ice. Following a wash in SB, the secondary goat anti-mouse $F(ab')_2$ fluorescein isothiocyanate-conjugated antibody was diluted 1:100 in SB and incubated for 30 min on ice. The cells were then washed and resuspended in 1 ml of SB and placed on ice for analysis by flow cytometry.

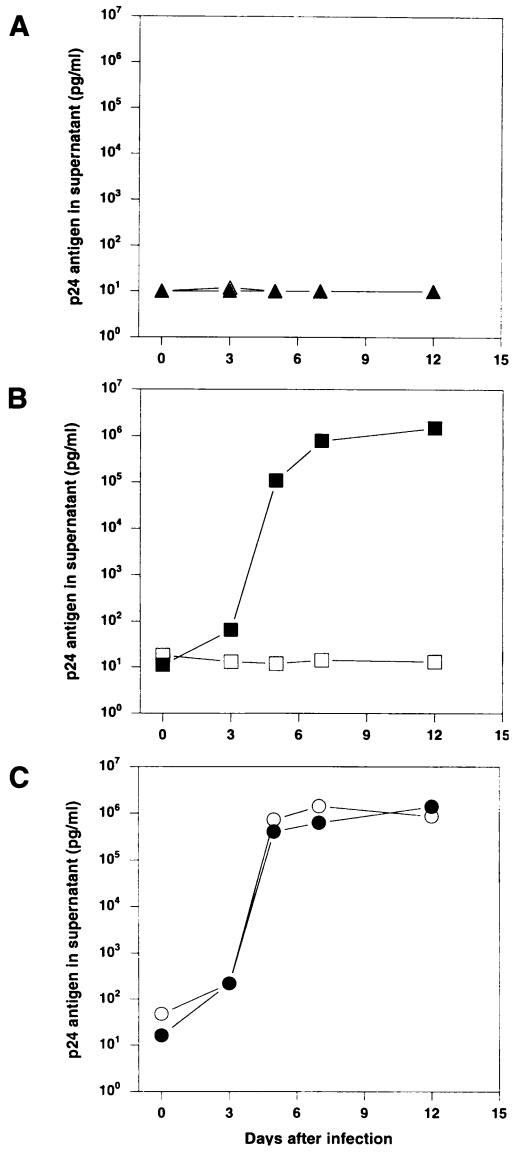

FIG. 1. Replication of HIV-1(IIIB) and HIV-1(89.6) in parental and hybrid cell lines. Replication of HIV-1(IIIB) (closed symbols) or HIV-1(89.6) (open symbols) was determined in 721.174 (A), CEM.3 (B), and CEMx174 (C) cells by measuring p24 antigen production in the extracellular medium at the time points indicated. These results are representative of five independent experiments.

RESULTS

HIV-1(89.6) infection of CEMx174 cells. The CD4-positive hybrid CEMx174 cell line was derived from the fusion of the CD4-negative B-cell line 721.174 and the CD4-positive T-cell line CEM.3 (40). To determine whether HIV-1(89.6) could infect either parental cell line, we infected all three cell lines at an MOI of 0.01 $TCID_{50}$ per cell, as determined by the $TCID_{50}$ in CEMx174 cells. For comparison, we used the prototype T-cell line tropic isolate HIV-1(IIIB) (38). As shown in Fig. 1,

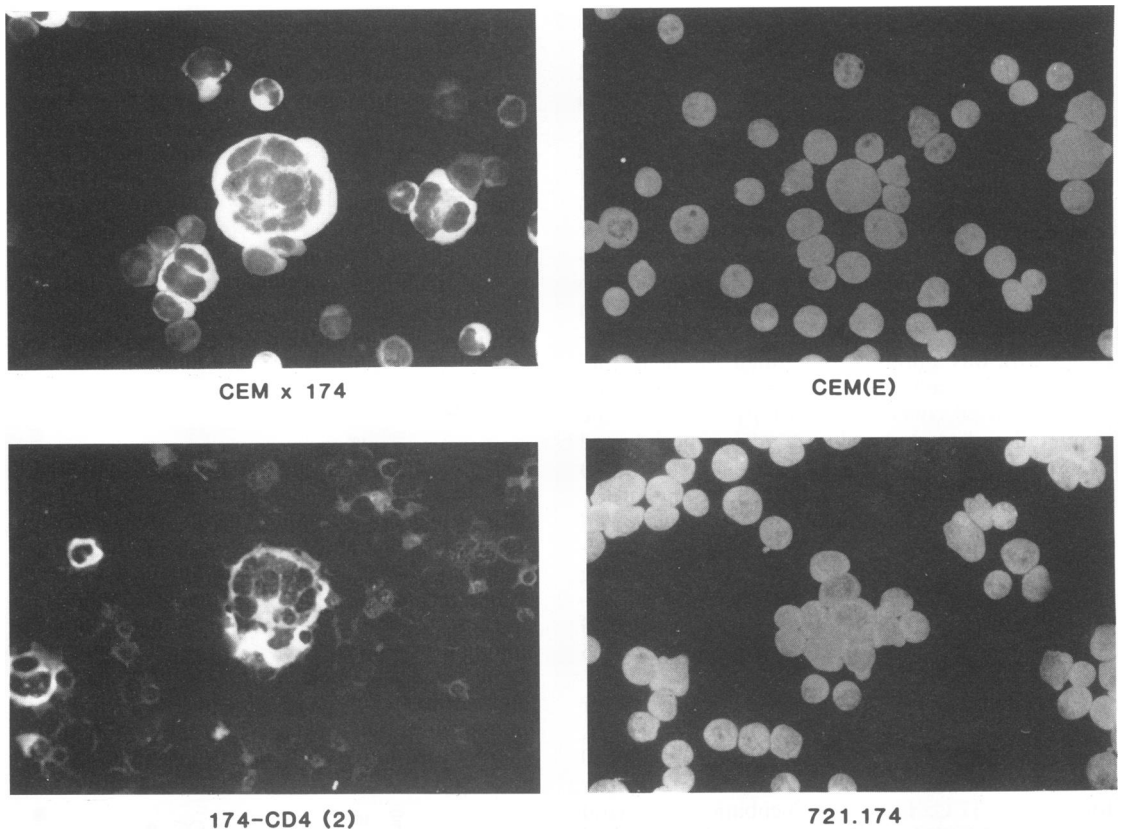

FIG. 2. IFA of cells infected with HIV-1(89.6). Cultures infected with HIV-1(89.6) were stained with a mixture of monoclonal antibodies directed against HIV-1 p24 and a conserved region of gp120 (see Materials and Methods). Approximately 90% of the CEMx174 cells infected with HIV-1(89.6) demonstrated intracellular expression of the viral proteins, while the parental cells, CEM.3 or 721.174, similarly exposed to virus, demonstrated only background fluorescence. Approximately 15 to 20% of the HIV-1(89.6)-infected 174-$CD4_2$ cells (721.174 engineered to express CD4) demonstrated fluorescence, corresponding with the flow cytometry analysis. Also illustrated are the prominent syncytia induced by HIV-1(89.6) in CEMx174 and, to a lesser degree, 174-$CD4_2$ cells.

HIV-1(89.6) replicated to high levels in CEMx174 cells, producing approximately 10^6 pg of $p24^{gag}$ antigen per ml in the supernatant by 7 days postinfection. This infection was accompanied by marked cytopathic effect, including syncytium formation (Fig. 2). However, HIV-1(89.6) did not replicate in either parental cell line at this MOI. In contrast, HIV-1(IIIB) replicated efficiently in both CEM.3 and CEMx174 cells but, as expected, not in the CD4-negative 721.174 cells.

Using IFA, we found that approximately 90% of CEMx174 cells infected with HIV-1(89.6) had robust intracellular expression of $p24^{gag}$ and gp120. Concordant with the absence of extracellular $p24^{gag}$ antigen, IFA of the CEM.3 and 721.174 cells infected with HIV-1(89.6) showed only background fluorescence (Fig. 2).

We then determined whether the block to infection of HIV-1(89.6) in CEM.3 cells could be overcome by increasing the inoculum. At MOIs of 0.1 and 1 $TCID_{50}$ per cell, low levels of $p24^{gag}$ antigen (100 to 1,000 pg/ml) could be detected in the supernatant of HIV-1(89.6)-infected CEM.3 cells. However, at all MOIs, the level of viral antigen produced was at least 1,000-10,000-fold less than that produced by HIV-1(IIIB) in CEM.3 cells or by either strain in CEMx174 cells (Fig. 3). Thus, the relative restriction of HIV-1(89.6) in CEM.3 cells remained consistent, even though at higher MOIs.

HIV-1(89.6) infection of CEMx174 cells is CD4 dependent. Since HIV-1(89.6) could productively infect the CD4-positive

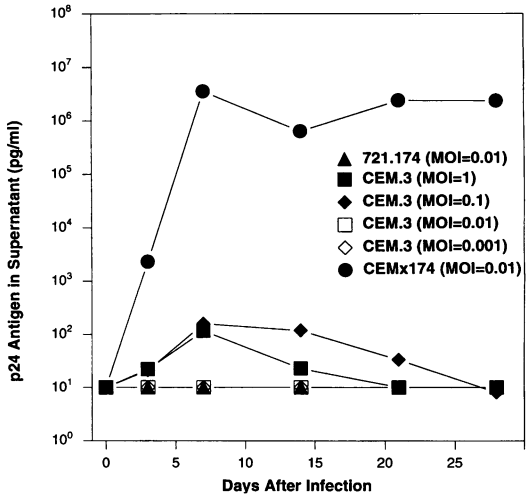

FIG. 3. HIV-1(89.6) infection of CEM.3 cells at different MOIs. Replication of HIV-1(89.6) in CEM.3 cells was determined by the production of $p24^{gag}$ antigen in the supernatant after infection at four different MOIs. Replication of HIV-1(89.6) in CEMx174 and 721.174 cells after infection at an MOI of 0.01 in the same representative experiment is shown for comparison.

TABLE 2. Expression of CD4 in lymphoblastoid cell lines, as determined by the mean channel fluorescence intensity of parental, hybrid, and retroviral vector-infected cells[a]

Cell line[b]	CD4 expression	Mean channel fluorescence[c]			
		Leu3a	W6/32	Leu16	CX2.1
CEMx174	Yes	224	738	578	271
CEM.3	Yes	325	121	—	—
721.174	No	0	512	909	—
174-G1N	No	—	382	671	2
174-$CD4_2$	Yes	13	412	671	2
174-$CD4_{2S}$	Yes	28	401	807	0

[a] Cells were stained with monoclonal antibodies against CD4 (Leu3a), MHC class I antigens (W6/32), an early B-cell surface marker (Leu16), or MHC class II (HLA-DR) antigens (CX2.1).

[b] Data for three lines of 721.174 cells infected with a retroviral vector are shown: 174-$CD4_2$, infected with a vector expressing CD4 and neomycin resistance but unselected; 174-$CD4_{2S}$, selected for high expression of CD4; and 174-G1N, infected with a vector that confers resistance to neomycin alone.

[c] In each instance, mean channel fluorescence intensity has been corrected by subtracting the reading with a control hybridoma supernatant. —, below the control hybridoma supernatant level. See Materials and Methods for details.

TABLE 3. Transfection of HIV-1(89.6) proviral DNA into 721.174 and CEM.3 cells results in viral replication[a]

Cell line	$p24^{gag}$ antigen in supernatant			pCDNA3/CAT sp act (cpm/μg of protein)[b]
	Level (pg/ml)[c]		Ratio, p89.6/pHXB2D	
	p89.6	pHXB2D		
CEM.3	1,857	2,092	0.89	88
CEMx174	1,524	1,809	0.84	807
721.174	681	1,161	0.59	541

[a] Infectious proviral DNA from either HIV-1(IIIB) (pHXB2D) or HIV-1(89.6) (p89.6) was transfected into 721.174, CEM.3, and CEMx174 cells by using DEAE-dextran (see Materials and Methods). As a control, the cells were transfected in parallel with pCDNA3/CAT, which expresses the CAT gene under the control of the CMV early promoter. The data are from one experiment and are representative of three independent experiments.

[b] Calculated 48 h after transfection, using a liquid scintillation assay (see Materials and Methods).

[c] Determined 48 h after transfection.

hybrid CEMx174 cell line but not the CD4-positive parental CEM.3 cell line, it was important to determine whether HIV-1(89.6) utilized surface CD4 as its receptor for infection of CEMx174 cells, as it does for infection of MDM and PBLs (7). Preincubation of HIV-1(89.6) with 50 μg of rsCD4 per ml completely blocked infection of CEMx174 cells (data not shown). Similarly, preincubation of the cells with Leu3a, a monoclonal antibody that recognizes the gp120 binding site on CD4 (41), resulted in inhibition of viral replication (data not shown).

We also determined the levels of CD4 in both CEM.3 and CEMx174 cells to rule out the possibility that the differences in replication could be explained on the basis of levels of CD4 expression. By flow cytometry, close to 100% of each cell type expressed CD4, and the mean channel fluorescence intensity was usually higher in CEM.3 than in CEMx174 cells (Table 2). Similar results have been published previously (26).

The restriction for HIV-1(89.6) in the parental cell lines lies at the level of viral entry. The ability of HIV-1(89.6) to replicate only in the hybrid cell line and not in the parent T-lymphoid line, in spite of equivalent levels of surface CD4, suggested that there must be additional cellular factors necessary for productive infection by HIV-1(89.6). These factors could be at the cell surface, facilitating CD4-dependent entry of the virus, or they could be involved in postentry events, perhaps enhancing the efficiency of reverse transcription or proviral expression. To distinguish between these possibilities, three experiments were performed. In the first, each of the cell lines was transfected with the molecular clones of strains 89.6 and IIIB, and viral expression was determined by supernatant $p24^{gag}$ antigen production (Table 3). At 48 h after transfection, expression of the two proviral clones was similar in each of the cell lines, in spite of a significantly higher apparent transfection efficiency for the CEMx174 and 721.174 cell lines, as determined by parallel transfections with pCDNA3/CAT. After 48 h, there was a marked increase in the level of replication in the CEMx174 cells, resulting from subsequent rounds of infection; neither the parental CEM.3 nor the 721.174 cells were able to sustain long-term viral production. To confirm that the rise in $p24^{gag}$ corresponded to infectious virus production, samples obtained 48 h after transfection were assayed in CEMx174. The levels obtained from all three transfections with each virus were comparable (data not shown).

In the second set of experiments, we examined virus uptake into the host cell. The parental and hybrid cell lines were incubated with either HIV-1(IIIB) or HIV-1(89.6) at either 0 to 4 or 37°C. Incubation at 4°C would allow virus binding only, while incubation at 37°C would permit virus uptake (22). Following incubation, virus that had not been internalized into the cells was removed by trypsin treatment, and the cells were lysed (see Materials and Methods). Assaying for the presence of viral $p24^{gag}$ antigen indicated the amount of virus that was taken up by each cell line. As depicted in Fig. 4, HIV-1(89.6) was efficiently internalized by the CD4-positive hybrid CEMx174 cell line at 37°C, not at 0°C, but was poorly internalized by either of the two parental cell lines at either temperature. In contrast, the control virus HIV-1(IIIB) was efficiently internalized by both the CD4-positive hybrid CEMx174 and parental CEM.3 cell lines at 37°C, although, as expected, not by the CD4-negative parental 721.174 cell line.

To further demonstrate that virus entry of HIV-1(89.6) into the CEMx174 cell line was CD4 dependent, these experiments were repeated following preincubation of the cells with Leu3A, an anti-CD4 monoclonal antibody. Internalization of HIV-1(89.6) was inhibited by Leu3A but not by either the negative control antibody or by PBS (data not shown).

In a third series of experiments, we looked at the initial reverse transcription of either HIV-1(89.6) or HIV-1(IIIB) in the three cell lines, taking advantage of the strand-switching event characteristic of retroviral reverse transcription. Primers encompassing the LTR were designed to amplify DNA from either virus (with size differences) and used in 16-h infections at an MOI of 0.01 $TCID_{50}$ per cell (34). As shown in Fig. 5, these primers amplified a specific band from infections with either virus in CEMx174 cells. The specificity of the bands was confirmed by Southern blotting using an LTR-specific probe (data not shown). However, only HIV-1(IIIB) was reverse transcribed in the CEM.3 line. As expected, heat-inactivated virus was not infectious, and specific signals were not obtained from 721.174 cells inoculated with either HIV-1 strain. Taken together, these results indicated that at least one block for HIV-1(89.6) in the parental cell lines must lie at the level of virus entry into the cell.

721.174 cells expressing CD4 are permissive for HIV-1(89.6) infection. These results suggested that a cellular factor important for efficient entry was expressed by the CEMx174 hybrid cell line but not by the CEM.3 parent cell line, which is CD4 positive. A logical prediction was that the B-cell line, 721.174, was contributing an additional factor(s) important in HIV-

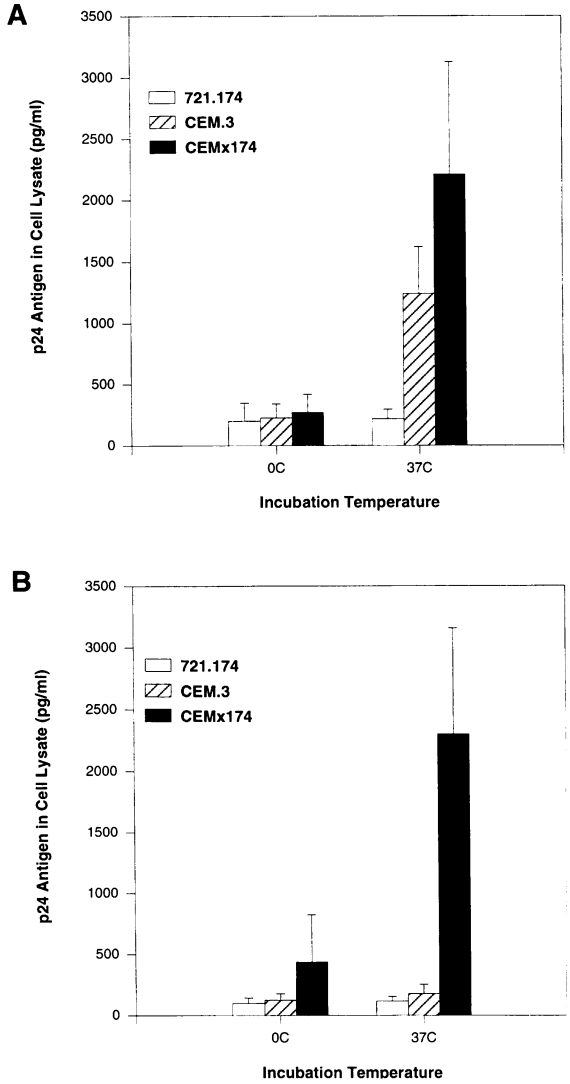

FIG. 4. Internalization of HIV-1 strains into parental and hybrid cell lines correlates with infectability. Cells were incubated with HIV-1 at 37°C for 1 h to allow virus binding and internalization, treated with trypsin, and lysed. The cell lysates, representing cytoplasmic viral antigen, were assayed for the presence of p24 antigen. For each strain of HIV-1 (89.6 or IIIB), the data represent the mean values ± 1 standard deviation from five independent experiments. Panels: A, HIV-1(IIIB); B, HIV-1(89.6).

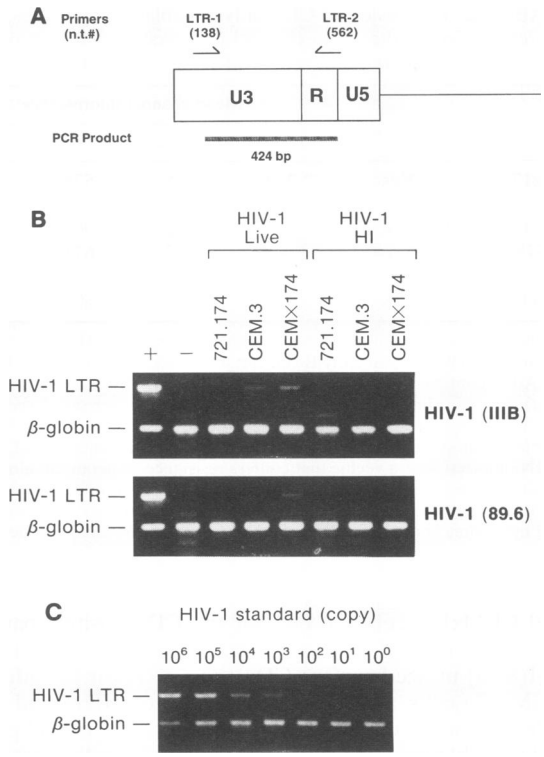

FIG. 5. Amplification of LTR sequences early in HIV-1 infection. (A) LTR-specific primers were used to amplify early products of reverse transcription as described in Materials and Methods. n.t., nucleotide. (B) Sixteen hours after infection at an MOI of 0.01 with either live or heat-inactivated (HI) virus, the cellular DNA was extracted and amplified with LTR and β-globin primers. Positions of the expected products are indicated for HIV-1(IIIB) and HIV-1(89.6). A chronically infected CEMx174 culture was used as positive control (+), and uninfected cellular DNA was used as a negative control (−). (C) The HIV-1-infected ACH-2 line, which contains a single copy of the HIV-1(IIIB) genome, was used to estimate the level of sensitivity of the PCR protocol. Serial 10-fold dilutions of ACH-2 cells diluted in CEMx174 cells to a total count of 10^6 cells are indicated. A signal was obtained when ACH-2 cells were diluted 1:1,000 in uninfected CEMx174 cells (10^3 per lane).

1(89.6) entry. To address this possibility, we constructed a 721.174 cell line expressing the CD4 molecule. This cell line expressed CD4 at levels easily measurable by flow cytometry (Table 2), though lower than levels expressed by the CEM.3 and the CEMx174 cell lines. Figure 6 shows that 721.174 cells expressing surface CD4 (174-$CD4_{2S}$) were permissive for HIV-1(89.6), reaching high levels of $p24^{gag}$, with plateaus similar to those of CEMx174. A slight delay in kinetics may be explained by the lower levels of CD4 in the 174-$CD4_{2S}$ cell line.

DISCUSSION

We have demonstrated that an M-tropic isolate, HIV-1(89.6), can efficiently infect the CD4-positive T-cell/B-cell hybrid line CEMx174, while it does not replicate well in either of its two parent cell lines, even though one expresses high levels of CD4. We suggest that despite the fact that CD4 is its receptor, one block for HIV-1(89.6) in the CEM.3 line is in the initial steps of the infectious process. A potential explanation for these results is that the parent T-cell line, CEM.3, expresses an inhibitor to HIV-1(89.6) entry. Equally plausible is the alternative hypothesis that the B-cell parent is contributing a factor which, in conjunction with CD4, allows efficient internalization of the M-tropic strain. This additional cellular factor(s) could either be a cell surface component, serving as an accessory molecule for entry, or be responsible for differences in membrane fluidity and fusion or other postbinding/fusion events of viral entry. This putative accessory factor(s) is most likely not an absolute requirement for HIV-1(89.6) entry, since at high MOIs, the virus replicated to low levels in CEM.3 cells. Alternatively, this factor could also be expressed by both parents but exist at higher levels in the B-cell parent and in the hybrid cell. It is important to emphasize that the putative accessory factors present in the B-cell/T-cell hybrid are unlikely to be those universally responsible for M tropism. A limited series of experiments indicated that CEMx174 cells are

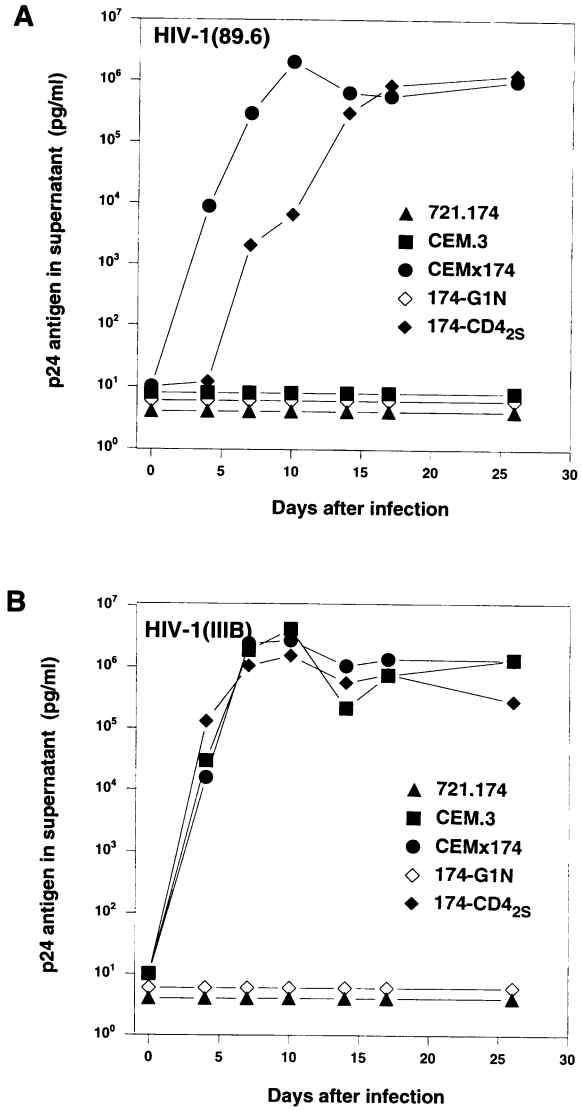

FIG. 6. Replication of HIV-1(IIIB) and HIV-1(89.6) in 174-CD4$_{2S}$ cells, a clone of 721.174 cells engineered to express CD4. Replication of HIV-1(89.6) (A) or HIV-1(IIIB) (B) after infection at an MOI of 0.01 was determined for 721.174, CEM.3, CEMx174, 174-G1N, and 174-CD4$_{2S}$ cells by measuring p24^{gag} antigen production in the extracellular medium at the time points indicated. These results are representative of at least two independent experiments.

not permissive for all M-tropic strains (data not shown), and thus the cell line cannot serve as a surrogate for primary macrophages. However, identification of a mechanism that facilitates HIV-1 entry into CD4-positive cells may lead to a better understanding of the entry process in primary cells like MDM and PBLs, even if the specific cellular factor(s) involved are different.

Our results are consistent with studies of other M-tropic strains of HIV-1, which are generally difficult to grow in continuous cell lines, leading to the suggestion that an accessory entry molecule is present in macrophages and PBLs but not in continuous cell lines, even those that are monocyte derived (8, 9, 18, 34). This hypothesis has been strengthened by genetic mapping experiments demonstrating that the inability of M-tropic strains to infect T-cell lines can be abrogated by replacement of certain portions of *env* with homologous fragments from L-tropic strains (2, 3, 27, 36, 44, 45, 48, 49). The active fragments do not include the domain of *env* associated with binding to CD4, in concordance with the observations that the CD4 molecule is the principal receptor in MDM as well as PBLs and that M tropism has not been associated with major differences in the affinities between gp120 and CD4 (28). Those data, along with the observations reported here, suggest that *env* domains not involved in CD4 binding may interact with accessory cellular molecules in as yet poorly defined steps in viral entry.

A number of molecules have been implicated as potential facilitators of entry for HIV-1. Among these are adhesion molecules, including the lymphocyte function-associated antigen 1 and the intercellular adhesion molecule 1 (20, 23, 29, 37, 46, 47). These studies have used only L-tropic HIV-1 strains, and further investigations using M-tropic strains of HIV-1, including strain 89.6, are clearly warranted. The major histocompatibility molecules have also been implicated in HIV-1 infection (10, 50). However, we do not feel that these molecules play a simple role in HIV-1(89.6) infection, since we dissociated both class I and class II expression from this phenotype (Table 2).

While infection with HIV-1 begins with binding of the viral surface glycoprotein gp120 to CD4, the events that follow in HIV-1 viral entry are not well defined. However, it is well accepted that expression of CD4 alone is not always sufficient for HIV-1 entry (4, 33). We have described a system in which a B-cell line expresses a determinant critical for efficient entry of an M-tropic strain. This model will allow us to map the viral determinants responsible for this phenotype and more importantly may also serve to identify the cellular factor(s) involved in mediating entry of HIV-1(89.6) into CD4-positive cells.

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