Human Immunodeficiency Virus Pseudotypes with Expanded Cellular and Species Tropism

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One mechanism for expanding the cellular tropism of a virus is through the formation of phenotypically mixed particles or pseudotypes, a process commonly occurring during viral assembly in cells infected with two or more viruses. We report here that dual infection of cells with human immunodeficiency virus (HIV) and a murine amphotropic retrovirus leads to the production of HIV pseudotypes that have acquired the host range of the amphotropic retrovirus and are capable of infecting not only CD4⁺ human cells but also mouse cells. The replication of the HIV pseudotypes in the various CD4⁺ cells was determined by measuring the appearance of HIV antigens in the supernatants, by cocultivation of CD4⁺ CEM cells with the infected CD4⁺ cells, and in some cases by assaying the culture supernatants directly for infectious virus. Of the cells tested, human foreskin fibroblasts were the best host cells, and by *in situ* cytohybridization, we were able to document that all cells in the culture were infected. In addition, the temporal appearance of HIV-specific proteins in the HIV pseudotype-infected fibroblasts was similar to that seen in CD4⁺ CEM cells. If the human fibroblasts were first infected with the amphotropic retrovirus, they demonstrated the property of superinfection exclusion and were resistant to subsequent infection by the HIV pseudotype. In other cell lines, including the human glioblastoma-derived cell line U373MG, HeLa cells, BALB/c mouse embryo cells, and SC-1 wild mouse cells, although the HIV pseudotype infection appeared to be less efficient, substantial amounts of HIV were nevertheless produced. These results indicate that the HIV (amphotropic retrovirus) pseudotypes may be useful for studying the molecular biology of HIV infections in a wide range of cells.

Human immunodeficiency virus (HIV) has been implicated as the etiologic agent of acquired immune deficiency syndrome (AIDS). This virus, a member of the retrovirus family, initiates infection through the interaction of its envelope glycoprotein, gp120, with the cellular receptor, CD4 (11, 25, 27, 28, 33, 35, 36). The experiments showing that HIV can preferentially replicate in and kill or impair subsets of lymphocytes, monocytes, and macrophages expressing CD4 on their cell surfaces have provided support for the hypothesis that the immunological manifestations of AIDS might be directly related to such infections *in vivo* (for a review, see reference 14). Of recent interest, however, are the *in vivo* and *in vitro* studies which suggest that HIV may be able to enter and replicate in cells which express no detectable CD4, including B cells, bone marrow precursor cells, muscle cells, glial cells, astrocytes, oligodendrocytes, neuronal cells, endothelial cells, squamous cell carcinoma cells, colorectal cells, bowel enterochromaffin cells, and fibroblasts (2, 6–8, 10, 13, 16, 20, 21, 26, 30, 32, 40, 43, 45, 47, 50, 52, 54, 57). This extended host range of HIV gains increasing importance in view of the broad spectrum of clinical disease seen in HIV-infected individuals. The pathogenic mechanisms underlying these multiple disease manifestations remain unknown, but it is very likely that HIV infection of CD4⁺ cells will be involved, at least in part.

The above considerations have focused attention on the need to develop model systems for studying the molecular biology of HIV replication in a broad range of host cells. One difficulty, however, in studying HIV replication in CD4⁺ cells is that *in vitro*, such infections are relatively inefficient due to inhibition at the adsorption step. Since molecular

analysis of HIV infections in these cells requires that the majority of the cells in the culture be infected, we sought a means to obtain efficient HIV infection of a wide range of CD4⁺ cells. We reasoned that because the tropism of HIV is determined primarily by a specific interaction between the HIV gp120 envelope protein and the cellular CD4 molecule, the host range of HIV could be expanded, albeit transiently, through the use of phenotypically mixed HIV virions (or pseudotypes). Pseudotype formation is a well-documented process which occurs when two different viruses infect the same cell, resulting in progeny virions with the genome of one virus and the structural proteins of the other virus (for a review, see references 3, 44, 56, and 59). For two unrelated but replication-competent viruses, the viral glycoproteins are usually the only components exchanged, and the pseudotype virions produced commonly contain a mosaic of the viral glycoproteins of both viruses. In this regard, previous experiments have shown that when cells infected with human T-cell leukemia virus types I and II (HTLV-I and HTLV-II) or HIV are superinfected with vesicular stomatitis virus, a proportion of the progeny vesicular stomatitis viruses are pseudotypes (9, 11, 33), although assays were not performed for the reciprocal pseudotypes (i.e., the HIV genome with vesicular stomatitis virus glycoproteins).

To determine whether HIV could form pseudotypes, we chose to use an amphotropic retrovirus as the coinfecting virus. Amphotropic retroviruses are a class of broad-host-range viruses isolated from wild mice (*Mus musculus*) which replicate well in human, primate, feline, canine, guinea pig, rabbit, rat, and mouse cells (22, 46). We used one of the isolates, designated 4070A, to infect CEM cells, a CD4⁺ T-helper cell line. The persistently infected CEM cells are designated CEM-1B, and since the initial amphotropic virus,

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4070A, induces cytopathology in CEM cells while the virus being produced by the persistently infected CEM-1B cells does not, we have designated the virus produced by CEM-1B cells as Amphi-1B. Our results demonstrate that infection of a cell with both HIV and Amphi-1B generates HIV progeny that can efficiently infect and replicate in a broad range of CD4⁺ cells. The implications of these findings with respect to the use of HIV pseudotypes in model systems as well as their potential biological role in the pathogenesis of AIDS are discussed.

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MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (FF cells) were prepared as described by Tamashiro et al. (51) and were maintained in Dulbecco modified Eagle medium (DMEM; Irvine Scientific) supplemented with 10% fetal bovine serum (FBS). BALB/c mouse embryo cells (MEC) were prepared as previously described (37) and were maintained in DMEM supplemented with 10% calf serum. NIH 3T3 TK⁻ cells (55), PA12 cells (39), and wild mouse SC-1 cells (obtained from Martin Haas) were maintained in DMEM supplemented with 10% FBS. The human glioblastoma-derived cell line U373MG (ATCC HTB17) was a gift from Robert LaFemina and was maintained in DMEM-high glucose (4.5 g per liter) containing 10% FBS. HeLa cells were maintained in DMEM plus 10% newborn calf serum. HeLa T4⁺ cells which express the CD4 molecule (33) were obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, through a contribution from Richard Axel and were maintained in DMEM supplemented with 10% newborn calf serum and 600 µg of G418 per ml. CEM cells, a continuous human lymphoblastoid cell line of greater than 95% CD4⁺ cells, were maintained at 5×10^5 cells per ml in RPMI 1640 medium containing 10% FBS. All media were supplemented with 0.29 mg of L-glutamine per ml, 0.2 mg of penicillin-streptomycin per ml, 1.5 µg of Fungizone per ml, and 50 µg of gentamycin per ml.

CEM-1B cells are neomycin-resistant CEM cells that were selected in the presence of 600 µg of G418 per ml after infection of the cells with a mixture of a defective retrovirus vector carrying the DNA sequence from the Tn5 gene for neomycin resistance (designated Neo) and the replication-competent retrovirus 4070A (22, 46). The defective retrovirus vector is a modification of the Cistor Neo vector described by Sorge et al. (49). Cistor Neo contains most of the *cis*-acting components of the retrovirus 4070A necessary for viral replication and packaging but lacks the genes encoding viral proteins. Instead, this vector contains the DNA sequence Neo. This vector was modified by converting the *Cla*I site 3' of Neo to an *Xba*I site. The packaging cell line PA12 (39) was transfected by the calcium phosphate procedure (19) with the modified Cistor Neo vector, and 48 h posttransfection, transient virus was used to infect NIH 3T3 TK⁻ cells. Neomycin-resistant NIH 3T3 TK⁻ cells, selected in the presence of medium containing 300 µg of G418 per ml, were then superinfected with the amphotropic retrovirus 4070A, and filtered supernatants (0.45-µm filters) from these cells were used to infect CEM cells to generate CEM-1B cells. The amphotropic virus being produced by the persistently infected CEM-1B cells has been designated Amphi-1B.

The LAV strain of HIV, originally obtained from the Pasteur Institute, was propagated by low-multiplicity infection of CEM cells. HIV (Amphi-1B) pseudotype virus was obtained by low-multiplicity infection of CEM-1B cells. In a standard low-multiplicity infection, a 30-ml culture of CEM cells or CEM-1B cells at 5×10^5 cells per ml were infected with 0.5 ml of stock HIV containing approximately 1,000 to 3,000 ng of HIV antigens per ml. The amount of HIV antigen present in the HIV stocks was determined by using the Abbott HTLV-III antigen enzyme immunoassay (EIA) as described by the manufacturer (Abbott Laboratories). At 24 h postinfection (p.i.), the cultures were washed extensively to remove free virus and resuspended in fresh medium, and virus was harvested when the cells showed 100% cytopathic effect. CEM-1B and HeLa T4⁺ cells, 24 h prior to and during infection with HIV, were maintained in medium minus G418.

Immunoblotting. Infected or uninfected human FF cells were released from flasks with trypsin and were pelleted in a clinical centrifuge. CEM cells, infected or uninfected, were pelleted directly. All cell harvests were washed extensively with phosphate-buffered saline prior to preparation of cell lysates. Cell lysates were prepared, and samples (corresponding to approximately 3×10^5 cell equivalents) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (5% acrylamide-0.13% bisacrylamide stacking gel and a 10% acrylamide-0.13% bisacrylamide or a 12.5% acrylamide-0.103% bisacrylamide resolving gel) and transferred to nitrocellulose as described previously (58). The filters were placed in blocking buffer (Tris-buffered saline with 50 g of nonfat dry milk per liter, 0.1 ml of antifoam A emulsion [Sigma Chemical Co.] per liter, 0.02% NaN₃, 0.5% Nonidet P-40) at 22°C for 12 h and then incubated with one of the following antisera: (i) a 1:100 dilution of high-titer antisera from a patient with AIDS in blocking buffer for 24 h at 4°C; or (ii) a 1:1,000 dilution of mouse monoclonal antibody to the *gag* proteins p24, p40, and p55 (from Genetic Systems Corporation) plus a 1:1,000 dilution of goat polyclonal antibody to gp160 HTLV-III (produced under contract by Repligen and obtained from the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Disease, National Institutes of Health) in blocking buffer for 3 h at 22°C, followed by two rinses with Tris-buffered saline and incubation in a 1:1,000 dilution of rabbit anti-mouse immunoglobulin G and rabbit anti-goat immunoglobulin G (Jackson Immuno Research) in blocking buffer for 1 h at 22°C. The blots were rinsed twice with Tris-buffered saline, washed three times with blocking buffer for 30 min each time, and then incubated with 25 ml of 1 µCi of ¹²⁵I-labeled protein A (40 µCi/µg; ICN Pharmaceuticals Inc.) per ml in blocking buffer for 1 h at 22°C. Following this incubation, the blots were rinsed twice with Tris-buffered saline, washed three times with blocking buffer for 30 min each time, and then rinsed once with Tris-buffered saline and once with H₂O. The blots were then autoradiographed at -70°C with an intensifying screen.

Analysis of RNA from FF, HeLa T4⁺, and CEM cells. Polyadenylated total cellular RNA was prepared as described by Bradley et al. (4). Briefly, cells were washed with phosphate-buffered saline and suspended at a concentration of 10^7 cells per ml in lysis buffer containing 0.2 M NaCl, 0.2 M Tris hydrochloride (pH 7.5), 1.5 mM MgCl₂, 2% SDS, and 200 µg of proteinase K per ml. The lysate was passed through an 18-gauge needle three to five times to shear the DNA and then incubated for 2 h with intermittent agitation at 45°C. The oligo(dT) cellulose (0.1 g per 10^8 cells, type 3;

Collaborative Research) was hydrated in elution buffer (0.01 M Tris hydrochloride, pH 7.5) and equilibrated with binding buffer (0.5 M NaCl, 0.01 M Tris hydrochloride [pH 7.5]). After incubation at 45°C, the lysate was adjusted to 0.5 M NaCl, added to the oligo(dT) cellulose, and incubated with rocking for 20 min at room temperature. The cellulose was pelleted by low-speed centrifugation, the supernatant was poured off, and the cellulose was resuspended in fresh binding buffer; these washing steps were repeated two to three times. The washed cellulose was then added to an Isolab Dispo column (Isolab), and the packed cellulose was washed until the A_{260} of the eluate was less than 0.05. Poly(A)⁺ mRNA was eluted from the column with elution buffer and precipitated with two volumes of ethanol after adjusting the salt concentration to 0.3 M sodium acetate.

The RNA samples (5 µg) were subjected to formaldehyde gel electrophoresis. Agarose gels (1.2%) were cast in MOPS (morpholinepropanesulfonic acid) buffer (0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA [pH 7.0]) containing 0.66 M formaldehyde and were electrophoresed in the same buffer without the formaldehyde. RNAs were denatured in a solution containing 44% deionized formamide, 0.88× MOPS buffer, 0.02% SDS, 5 mM EDTA, 1.88 M formaldehyde, 6% glycerol, and 0.05% bromophenol blue at 65°C for 15 min. Before loading, a 1/25 volume of an ethidium bromide solution (1 mg/ml) was added to the RNA samples. After electrophoresis, the gel was photographed directly and then soaked for 20 min in 0.05 M NaOH–1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), followed by two 20-min washes in 10× SSC, and finally transferred to a nitrocellulose filter in 10× SSC by blotting. The filter was baked in a vacuum oven at 80°C for 2 h and prehybridized at 45°C for 16 h in a solution containing 50% formamide, 5× SSC, 50 mM NaPO₄ (pH 7.0), 10× Denhardt reagent, 250 µg of salmon DNA per ml, 4 mM EDTA, and 0.5% SDS. Hybridization was performed at 45°C in a solution containing 50% formamide, 5× SSC, 40 mM NaPO₄ (pH 7.0), 1× Denhardt reagent, 100 µg of salmon DNA per ml, 1 mM EDTA, 0.1% SDS, 10% dextran sulfate, and 2×10^6 cpm/ml of the ³²P-labeled CD4-specific probe prepared as described below. The blot was washed in 2× SSC–0.1% SDS at room temperature for 1 h, two times for 30 min each time in 0.1× SSC–0.1% SDS at 50°C, rinsed in 0.1× SSC at room temperature, and subjected to autoradiography at –70°C in the presence of intensifying screens.

The CD4-specific probe was prepared from the recombinant clone designated pT4B (34). This clone, which contains the cDNA encoding the CD4 receptor of human T lymphocytes inserted into the *Eco*RI site of the plasmid SP65, was obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Disease, National Institutes of Health, through a contribution from Richard Axel. The DNA was linearized by cleavage with *Eco*RI and labeled with [α -³²P]dCTP (3,000 Ci/mmol) by using the random-primed DNA-labeling kit according to the directions of the manufacturer (Boehringer Mannheim Biochemicals).

In situ cytohybridization. In situ cytohybridization was performed essentially as described by Gendelman et al. (18), with the following modifications: trypsinized FF and CEM cells (1.5×10^5 cells per slide) were washed with phosphate-buffered saline, resuspended in 30 µl of phosphate-buffered saline, deposited in a spot onto pretreated slides coated with 0.1% poly-D-lysine, and fixed with ethanol-acetic acid (3:1). ³⁵S-labeled hybridization probes (specific activity of 1×10^8 to 2×10^8 cpm/µg) were prepared by nick translation with

[³⁵S]dCTP as described previously (38). The hybridization mixture consisted of 50% formamide, 0.6 M NaCl, 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 1× Denhardt reagent, 10% dextran sulfate, 100 µg of poly(A) per ml, 250 µg of calf thymus DNA per ml, 1 mg of human placenta RNA per ml, 0.1% Triton X-100, 10 mM dithiothreitol, and 7×10^7 cpm of ³⁵S-labeled probe per ml. Hybridization was for 60 h at room temperature. After hybridization, the slides were washed as follows: twice for 5 min each time in 50% formamide buffer (50% formamide, 600 mM NaCl, 10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, and 0.1% Triton X-100) at room temperature; three times for 20 min each time in 2× SSC–0.1% Triton X-100 at 55°C; 24 h with three changes in 50% formamide buffer at room temperature; and twice for 5 min each time in 2× SSC–0.1% Triton X-100 at room temperature. The slides were then dehydrated in graded ethanols, dipped in emulsion, exposed, developed, and stained with Giemsa stain.

The ³⁵S-labeled HIV probe was prepared by using an 8,930-nucleotide *Sst*I fragment from the HIV proviral clone H9pv22 (kindly provided by Cirilo Cabradilla); this clone was isolated from chronically infected H9/HTLV-III cells which contain several integrated copies of HIV (41). Negative controls were performed with the plasmid vector pACYC184 (5).

Biosafety. All experiments involving live HIV were performed at the highest level of containment in a locked BL3 facility, and there was strict adherence to established National Institutes of Health procedures for research conducted at the BL3 level of containment.

RESULTS

Generation of HIV pseudotypes. Our strategy for producing HIV pseudotypes was to use a CD4⁺ cell line that was persistently infected with an amphotropic retrovirus. This cell line, designated CEM-1B, was generated as described in Materials and Methods. Because it was possible that the presence of the amphotropic retrovirus might interfere with the replication of HIV, we first examined the kinetics of HIV synthesis in both CEM-1B cells and in a parallel culture of the parental CEM cells. For this experiment, CEM and CEM-1B cells were infected with a low multiplicity of the LAV strain of HIV and were monitored daily for the production of HIV in the supernatants by using the Abbott HIV antigen EIA (data not shown). We noted that the HIV infection in the CEM-1B cells progressed more rapidly than that in the CEM cells. Beginning on day 6 p.i., there was a small increase in HIV antigen production, and its level continued to increase until day 11, when it was 34 times higher in the supernatants from the HIV-infected CEM-1B cell line than in the supernatants from the HIV-infected CEM cells. Two days later, virus production in the HIV-infected CEM cells peaked and was approximately equivalent to that seen for the infected CEM-1B cells. Cytopathology, as indicated by syncytia formation and cell death, was also accelerated in the HIV-infected CEM-1B cells. In both cultures, most of the cells died, but a population of persistently infected cells producing comparable titers of HIV and showing no obvious cytopathology survived.

Pattern of replication of HIV pseudotypes in CD4⁺ human fibroblasts. To ascertain whether the HIV-infected CEM-1B cultures were generating HIV pseudotypes, we first determined whether the HIV progeny had acquired the host range of the amphotropic virus and could infect CD4⁺ human cells. For this experiment, we used primary FF cells. To document

FIG. 1. Northern (RNA) analysis of CD4-specific mRNA in CEM cells (lane 1), FF cells (lane 2), and HeLa T4⁺ cells (lane 3). Polyadenylated total cellular RNA was electrophoresed on a denaturing formaldehyde gel, transferred to nitrocellulose, and hybridized with a ³²P-labeled CD4-specific probe as described in the Materials and Methods.

that the cells were CD4⁻, polyadenylated RNA was isolated from the FF cells, as well as from CEM and HeLa T4⁺ cells (33), as positive controls. The RNAs were subjected to electrophoresis through denaturing formaldehyde gels, transferred to nitrocellulose, and hybridized with a CD4-specific probe prepared from the recombinant clone designated pT4B (34). No CD4-specific RNA could be detected in the sample from FF cells (Fig. 1). In contrast, CD4-specific transcripts were readily detected in the two positive controls. In CEM cells, an endogenous mRNA of 3.0 kilobases was present, while in the HeLa T4⁺ cells, the size of the transcript was 6.3 kilobases. This larger RNA is consistent with the predicted size of a transcript generated from the recombinant clone used to produce the HeLa T4⁺ cells (24).

In the experiment shown in Fig. 2, the CD4⁻ FF cells and CD4⁺ CEM cells were infected with filtered supernatants either from CEM-1B cells infected with HIV or from the parallel culture of HIV-infected CEM cells. As a control, we also mixed the supernatant from the HIV-infected CEM cells containing only HIV with supernatant containing only Amphi-1B virus prior to infection of FF cells. At various time intervals p.i., HIV production was monitored by the appearance of HIV antigens in the supernatant. Both sources of HIV were able to infect and replicate to high titers in CD4⁺ CEM cells. As expected, the CD4⁻ FF cells were resistant to infection with HIV that had been passaged through CEM cells, even when mixed with amphotropic retrovirus just prior to infection of the FF cells. However, consistent with the hypothesis that HIV pseudotypes were being produced by the HIV-infected CEM-1B cells, HIV in the supernatants from these cultures was able to infect and replicate in the FF cells (Fig. 2). Furthermore, filtered supernatants harvested from the HIV pseudotype-infected FF cultures at day 20 contained cell-free virus capable of infecting CD4⁺ CEM cells (data not shown). The control cultures of uninfected FF

FIG. 2. Permissive infection of FF cells by HIV (Amphi-1B) pseudotype. Cell-free supernatants (filtered through 0.45- μ m filters) from CEM cells and CEM-1B cells infected with HIV were used as a source of HIV and HIV (Amphi-1B) pseudotypes, respectively, for infecting fresh CEM or FF cells. In this experiment, 5-ml cultures of CEM cells (5×10^5 cells per ml) or T75 flasks of FF cells (2×10^6 cells per flask) were infected with 1 ml of HIV or HIV (Amphi-1B) pseudotype (each stock contained approximately 2,000 ng/ml of HIV antigens). For the experiment in which HIV was mixed with the Amphi-1B virus prior to infection of FF cells, 1 ml of cell-free supernatant from CEM-1B cells (containing Amphi-1B virus) was mixed with 1 ml of HIV. At various time intervals p.i., supernatants were obtained and the amount of HIV present was determined by using the HIV antigen EIA. Samples containing less than 50 pg/ml of HIV antigen were below the limits of sensitivity.

cells and FF cells infected with Amphi-1B were always negative in these assays.

In parallel with the above experiments, we also examined the temporal appearance of the HIV-specific proteins in HIV pseudotype-infected FF cells and CEM cells. To determine the intracellular steady-state concentration of the proteins throughout the infection, cell extracts from HIV pseudotype-infected FF and CEM cells at various time intervals p.i. or from uninfected cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were detected by immunoblotting with antisera from a patient with AIDS as described in Materials and Methods (Fig. 3A). At 8 h p.i., some *gag*-specific proteins (p55, p40, and p24) appeared to be present in the infected FF cells. However, due to the presence of low-abundance, cross-reactive proteins in uninfected FF cells which migrated at the same position as the p55 and p40 precursor *gag* proteins, we could not determine whether the p24 *gag* proteins detected were due to input virus or newly synthesized proteins. However, by 24 h p.i., in both the HIV pseudotype-infected FF cells and CEM cells, the envelope glycoproteins and *gag*-specific proteins were readily visualized. These proteins increased in abundance between 24 and 48 h p.i. and remained at approximately the same steady-state concentration through day 8. As a control (Fig. 3B), we

FIG. 3. Immunoblot analysis of cell lysates from uninfected and infected FF and CEM cells (A) Lysates (3×10^5 cell equivalents) obtained from uninfected FF or CEM cells or cells infected with HIV (Ampho-1B) pseudotypes for 8, 24, 48, or 72 h or for 5 and 8 days were separated by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide–0.103% bisacrylamide resolving gel), transferred to nitrocellulose, probed with antiserum from a patient with AIDS, and labeled with 125 I-protein A as described in the Materials and Methods. In this experiment, cultures (30 ml) of CEM cells at 5×10^5 cells per ml or T75 flasks of FF cells (2×10^6 cells per flask) were infected with 4 ml of a filtered stock of HIV (Ampho-1B) pseudotype virus (approximately 2,000 ng of HIV antigens per ml). At 24 h p.i., all cultures were washed extensively to remove free virus and resuspended in fresh medium. (B) Lysates (3×10^5 cell equivalents) obtained from uninfected FF cells or FF cells infected with HIV, HIV (Ampho-1B) pseudotype, or Ampho-1B virus for 7 days were separated by SDS-polyacrylamide (10% acrylamide–0.13% bisacrylamide resolving gel), transferred to nitrocellulose, probed with mouse monoclonal antibody to the *gag* proteins plus goat polyclonal antibody to gp160 HTLV-III followed by rabbit anti-mouse immunoglobulin G plus rabbit anti-goat immunoglobulin G, and labeled with 125 I-protein A as described in the Materials and Methods. In this experiment, T25 flasks of FF cells (3×10^5 cells per flask) were infected with 5 ml of filtered stock of HIV (approximately 3,000 ng per ml of HIV antigen), HIV (Ampho-1B) pseudotype (approximately 3,000 ng of HIV antigen per ml), or Ampho-1B virus (harvested from CEM-1B cells). At 1 day p.i., the cultures were washed extensively to remove free virus, and at 4 days p.i. the cultures were trypsinized and passaged 1:1 into T25 flasks.

show that at day 7 p.i. no intracellular HIV-specific *gag* or envelope proteins could be seen in FF cells infected with either HIV alone or with the amphotropic helper virus Ampho-1B.

Although the results of the above experiments suggested that the HIV pseudotypes could infect and replicate in the cultures of CD4⁺ FF cells, we could not eliminate the possibility that only a small percentage of the cells were infected. Therefore, to investigate the course of the infection at the cellular level and to determine whether all FF cells were infected with the HIV pseudotype, we harvested FF cells 7 days after high-multiplicity infection with HIV or HIV (Ampho-1B) pseudotypes and prepared them for in situ cytohybridization with a probe specific for HIV. Hybridizations were performed under conditions which would allow us to detect primarily HIV RNA. All FF cells were infected with HIV (Ampho-1B) pseudotypes (Fig. 4a). In contrast, on the entire slide containing 1.5×10^5 FF cells infected with HIV alone, there was only a single positive cell detected (Fig. 4c and d). As expected, uninfected FF cells (Fig. 4f) and FF cells infected with Ampho-1B (data not shown) showed no hybridization, and CEM cells persistently infected with HIV (Fig. 4h) were all positive. Additional control hybridizations with the labeled plasmid pACYC184 as the probe were negative in all cases (Fig. 4b, e, g, and i).

In the above experiments, we presented evidence that virus produced following HIV infection of CEM-1B cells

consisted of HIV pseudotypes capable of infecting CD4⁺ human fibroblasts. Since CEM-1B cells had been persistently infected with amphotropic retrovirus for a long period of time prior to infection with HIV, it was possible that the apparent production of HIV pseudotypes was due to some alteration in the CEM-1B cells rather than to the presence of the amphotropic retrovirus. To exclude this possibility and to monitor the kinetics of pseudotype formation, we used filtered supernatants from CEM-1B cells containing the amphotropic retrovirus Ampho-1B to infect CEM cells persistently infected with HIV. Filtered supernatants from these cultures, collected prior to or 3, 8, and 20 days after infection with Ampho-1B virus, were then used to infect human fibroblasts. At various time intervals p.i., the production of HIV in the FF cell cultures was assayed, and at day 20, CEM cells were added to the FF cultures to amplify the presence of low levels of HIV being produced. The results of these experiments demonstrated that the production of HIV pseudotypes (as measured by the ability to infect and replicate in human fibroblasts) was directly related to the establishment of the Ampho-1B infection in the CEM cells persistently infected with HIV (data not shown). By day 9 in the FF cultures, virus from the initial inoculum had been totally eliminated, and HIV could clearly be detected in the supernatants of the FF cultures exposed to the HIV progeny generated after 8 or 20 days of infection of the HIV persistently infected CEM cells with Ampho-1B virus. There were

FIG. 4. Photomicrographs of cells hybridized for RNA with an ^{35}S -labeled HIV probe (a, c, d, f, and h) or pACYC184 probe (b, e, g, and i). (a and b), HIV (Ampho-1B)-infected FF cells at 7 days p.i.; (c, d, and e), HIV-infected FF cells at 7 days p.i. (panel d shows a single infected cell); (f and g), uninfected FF cells; (h and i), HIV-infected CEM cells. The uninfected FF cells and FF cells infected with HIV or HIV (Ampho-1B) pseudotype are from the experiments described in Fig. 3B. The autoradiographic exposure time was 2 days, except for panel d (4-day exposure time). Original magnification, $\times 400$.

FIG. 5. Resistance of FF cells infected with Ampho-1B virus (FF-1B cells) to infection by HIV (Ampho-1B) pseudotype. T75 flasks of FF cells (approximately 2×10^6 cells per flask) were infected with 5 ml of filtered supernatant containing Ampho-1B virus from CEM-1B cells. The infected FF cells (designated FF-1B) were passaged four times over a period of 22 days to ensure complete spread of the Ampho-1B virus through the FF cell culture; uninfected FF cells were passaged and maintained in parallel. The uninfected FF cells and FF-1B cells were then infected with 3 ml of filtered supernatant containing HIV (Ampho-1B) pseudotype (approximately 1,000 ng of HIV antigens per ml), generated after passage of HIV through CEM-1B cells. Supernatants were assayed for the presence of HIV by using the Abbott HIV antigen EIA.

no detectable HIV antigens in the supernatants of FF cell cultures exposed to HIV virus alone or to the HIV progeny generated after only 3 days of infection of the CEM cells with Ampho-1B virus. However, although the production of HIV pseudotypes by cells which were infected with Ampho-1B virus for only 3 days was below the limits of detection as measured by the appearance of HIV antigens in the supernatants of the FF cell cultures (less than 50 pg/ml), we could find evidence for infectious HIV being produced by these FF cell cultures after cocultivation with CEM cells. In addition, we were able to demonstrate that cell-free HIV, infectious for CEM cells, was being produced from FF cells infected with the stock of HIV pseudotypes produced after 20 days of the Ampho-1B infection.

Blocking HIV pseudotype infection of human fibroblasts by prior infection with amphotropic retrovirus. As another test for the formation of HIV pseudotypes, we used an assay which relies upon the resistance of cells to infection by the virus pseudotype after infection with the heterologous virus. The basis of this assay is that infection with the heterologous virus (in this case Ampho-1B) will result in saturation of the cellular receptors with the Ampho-1B glycoproteins and, hence, infection of the CD4⁺ cell by the HIV pseudotype will be inhibited at the adsorption step. For this experiment, FF cells were infected with Ampho-1B and split four times over 22 days to ensure spread of the amphotropic retrovirus throughout the culture. These cells, designated FF-1B, and uninfected FF cell controls were then challenged with stocks of HIV which had been passaged through CEM-1B cells (thus presumably containing pseudotypes) or passaged through CEM cells. At various time intervals postchallenge, the supernatants from the FF cells were monitored for the production of HIV, as assayed by the presence of HIV antigens. Cultures preinfected with Ampho-1B resisted superinfection by the HIV pseudotypes, with approximately

100-fold less HIV antigens in the supernatant (Fig. 5). Consistent with previous experiments, both FF cells and FF cells preinfected with Ampho-1B were resistant to infection by HIV passed through CEM cells (data not shown).

Expansion of the cellular and species tropism of HIV. A final series of experiments was undertaken to determine the susceptibility of other CD4⁺ human and nonhuman cells to infection by the HIV (Ampho-1B) pseudotypes. We were particularly interested in assessing whether the apparent species-specific tropism of HIV was determined at the adsorption and penetration step and thus could be bypassed with the HIV (Ampho-1B) pseudotypes. Previous studies have shown that a variety of nonhuman cells transfected with a molecular clone of HIV produce infectious progeny virus, suggesting that there is no intracellular block to viral RNA or protein synthesis (1, 29). However, in these experiments, a barrier occurring subsequent to adsorption and penetration, but prior to transcription, would not have been detected, and precedent for such restriction exists in the murine retroviral system (53). HIV was passed through CEM-1B cells to generate a high titer of HIV pseudotypes. This stock of virus, as well as control HIV passed through CEM cells, was then used to infect FF cells, HeLa cells, HeLa T4⁺ cells, U373MG human glioblastoma cells, BALB/c MEC, and SC-1 wild mouse cells. At various time intervals p.i., the supernatants were assayed for the production of HIV, as measured by the presence of HIV antigens, and at day 20, CEM cells were added to the cultures to amplify the presence of low levels of HIV being produced. Tables 1 and 2 show that both stocks of virus replicated equally well in the HeLa T4⁺ cells and that all of the other cell types were susceptible to infection by the HIV pseudotypes, although virus production did vary considerably. FF cells were the best hosts, followed by the U373MG and HeLa cells. Less virus was produced by the murine cell cultures than by the human cultures; in the case of the SC-1 cells, virus production declined as the cells were passaged. In the above experiments, we also noted for the first time that FF cells as well as U373MG cells infected with a high titer of HIV (passed through the CEM cells) showed a low level of infection when CEM cells were added to amplify HIV, despite the lack of detectable HIV antigens in these cultures prior to cocultivation. These observations are consistent with those of others who have reported that at a high multiplicity of infection, HIV may be able to infect, albeit at a very low efficiency, CD4⁺ cells (2, 6-8, 10, 16, 21, 40, 47, 52). However, in these cases, the production of HIV can usually be detected only following cocultivation with CD4⁺ indicator cells. The possibility that these results were due to a small amount of residual virus from the initial inoculum seems unlikely because the cultures were repeatedly trypsinized, and in the other cell cultures (i.e., HeLa, MEC, and SC-1 cells), infection with HIV alone was not evident even when these cultures were cocultivated with CEM cells for extended periods of time. Control cultures, left uninfected or infected with Ampho-1B virus, also showed no evidence of HIV infection (data not shown).

DISCUSSION

In this report, we describe a method to obtain efficient HIV infection of a wide range of CD4⁺ cells. This was accomplished by first passaging HIV through CD4⁺ cells which were persistently infected with an amphotropic retrovirus, thus generating HIV pseudotypes. In general, when two unrelated enveloped viruses infect the same cell, there is

TABLE 1. Expanded tropism of HIV (Ampho-1B) pseudotypes

Infected cells, expt 1 ^a	HIV virus production (ng of HIV antigen per ml in the supernatant) at day p.i.					
	9	20	23	28	31	37
FF + HIV (Ampho-1B)	1,100	910	3,100	3,100	310	NA ^b
FF + HIV	0	0	0	14	7,300	NA
MEC + HIV (Ampho-1B)	7.6	5.8	210	3,400	485	NA
MEC + HIV	0	0	0	0	0	0
SC-1 + HIV (Ampho-1B)	2.0	0.07	7.5	5,600	820	NA
SC-1 + HIV	0	0	0	NA	0	0

^a Cells were plated at 3×10^5 cells per T25 flask and infected the following day with 2.5 ml of cell-free supernatant containing either HIV (Ampho-1B) pseudotype or HIV alone (each stock contained approximately 2,000 ng of HIV antigens per ml). Due to the different rates of cell growth for the different cell types, they were split at variable times as necessary. FF cells were maintained in T25 flasks and were not split. BALB/c MEC were transferred to T75 flasks on day 9 and were not additionally passaged. Wild-mouse SC-1 cells were transferred to T75 flasks on day 3 p.i. and split 1:4, 1:2, 1:8, and 1:6 on days 6, 9, 13, and 18, respectively. In all cultures, cells were trypsinized and washed extensively on day 3 p.i. to remove residual inoculum. The amount of HIV present in the supernatants was determined by using the Abbott HIV antigen EIA. On day 20 p.i., 4×10^4 CEM cells per ml in RPMI 1640 plus 10% FBS were added to amplify low levels of infectious HIV present in the cultures. On day 28 p.i., the nonadherent cells were removed from the initial flasks used in the cocultivation and maintained as suspension cultures.

^b NA, Not assayed.

phenotypic exchange of the envelope viral glycoproteins and the pseudotype progeny commonly contain a mosaic of the glycoproteins of both viruses (3, 44, 56, 59). It therefore seems likely that the HIV progeny contain Ampho-1B glycoproteins and depend upon the Ampho-1B receptor for efficient entry into CD4⁺ cells. However, because we do not have neutralizing antisera directed against the glycoproteins of the amphotropic retrovirus, we could not demonstrate this directly by serological assays. Nevertheless, the extended host range of the HIV pseudotypes coupled with the experiments showing that preinfection of the CD4⁺ FF cells with Ampho-1B markedly inhibit subsequent infection by the HIV pseudotypes provide strong support for the conclusion that the HIV pseudotypes do contain Ampho-1B glycoproteins.

In the experiments in which we compared the replication of HIV in the CEM cells with that in the CEM-1B cells, the presence of the amphotropic retrovirus did not appear to interfere with HIV replication. In fact, we noted that the infection in the CEM-1B cells was accelerated. One possible explanation for this result is that the amphotropic retrovirus stimulated HIV viral gene expression. Alternatively, HIV pseudotypes produced after the first round of replication may have been able to adsorb to or penetrate uninfected cells more efficiently, thus expediting the spread of HIV

through the culture. We believe that this latter possibility is more likely for the following reasons. The accelerated infection was more pronounced at low-input multiplicities of HIV, and peak titers produced by both cultures were the same. Furthermore, if we allowed the infected cultures to proceed past the point where most of the cells were killed, there arose in both cultures a population of persistently infected cells producing comparable titers of HIV. If the effect is due to hastened spread of the infection, there is still the question of mechanism. Since the HIV pseudotypes produced through the dual infection likely contain a mosaic of the HIV and amphotropic retrovirus glycoproteins, they could gain access to uninfected cells by using either the CD4 receptor or the receptor for the amphotropic retrovirus. However, as discussed above, FF cells infected with the amphotropic retrovirus demonstrate the property of superinfection exclusion. It is possible that there is a higher concentration of the receptor used by Ampho-1B on the CEM cells than on the FF cells. If this is the case, then infection of the CEM cells by Ampho-1B might produce an insufficient level of envelope proteins to saturate the receptor population. Alternatively, the presence of the Ampho-1B glycoprotein in the HIV envelope might potentiate the interaction of the HIV gp120 with the CD4 receptor. Clearly,

TABLE 2. Expanded tropism of HIV (Ampho-1B) pseudotypes

Infected cells, expt 2 ^a	HIV virus production (ng of HIV antigen per ml in the supernatant) at day p.i.					
	8	18	22	25	27	29
FF + HIV (Ampho-1B)	871	789	722	5,000	3,240	4,550
FF + HIV	0	0	0	63	4,930	5,000
HeLa (T4 ⁺) + HIV (Ampho-1B)	539	535	1,210	3,600	NA ^b	52
HeLa (T4 ⁺) + HIV	625	805	1,110	4,230	NA	49
HeLa + HIV (Ampho-1B)	66	84	10	2,200	2,410	243
HeLa + HIV	0.02	0	0	0	0	0
U373 MG + HIV (Ampho-1B)	114	135	564	2,610	500	209
U373 MG + HIV	0	0	0	6.5	233	5,000

^a Cells were plated at 3×10^5 cells per T25 flask and infected the following day with 5 ml of cell-free supernatant containing either HIV (Ampho-1B) pseudotype or HIV alone (each stock contained approximately 3,000 ng of HIV antigens per ml). FF cells were maintained in T25 flasks. HeLa cells were split 1:3, 1:5, 1:6, 1:6, and 1:6 on days 4, 8, 12, 15, and 19, respectively. HeLa T4⁺ cells were split 1:3 on day 4 p.i. U373MG human glioblastoma cells were split 1:3 on day 4 p.i., and the cells infected with HIV (Ampho-1B) pseudotypes were subsequently split 1:4, 1:4, and 1:2 on days 8, 12, and 19, respectively, while those infected with HIV alone were split 1:5, 1:4, 1:3, and 1:4 on days 8, 12, 15, and 19, respectively. In all cultures, cells were trypsinized and washed extensively on day 4 p.i. to remove residual inoculum. In addition to the above noted passages, the FF cells and HeLa T4⁺ cells were trypsinized and replated without splitting on day 8. On day 20 p.i., 5×10^5 CEM cells per ml in RPMI 1640 plus 10% FBS were added to amplify low levels of infectious HIV present in the cultures. On days 25 and 27 p.i., the nonadherent cells in the HeLa and HeLa T4⁺ cultures, respectively, were removed from the initial flasks and maintained as suspension cultures.

^b NA, Not assayed.

additional experiments will be necessary to resolve these various possibilities.

Individuals infected with HIV manifest a broad range of clinical disorders ranging from asymptomatic infection to full-blown AIDS. The progressive immunodeficiency of AIDS is characterized by depletion of CD4⁺ helper-inducer lymphocytes and is believed to result from the tropism of HIV for CD4⁺ lymphocytes, monocytes, and macrophages. Although immunodeficiency is a hallmark of the disease, patients infected with HIV may present with a large number of additional clinical problems, including neurologic impairment, gastrointestinal disorders, lymphoproliferative diseases, connective tissue disorders, retinitis, and thrombocytopenia. Many questions relating to these associated disorders remain unanswered. What are the cell types involved? Does HIV directly infect these cells or are the effects of the HIV infection indirect? If HIV does infect the involved cells, does it use the CD4-mediated entry pathway? And what is the role, if any, of other viral cofactors?

Recently, a great deal of attention has focused on the progressive neurologic dysfunction associated with HIV infection. HIV can be isolated from the brain or cerebrospinal fluid (23, 31), and in situ cytohybridization, electron microscopy, and antigen detection studies have revealed the presence of HIV gene products in brain cells (13, 17, 20, 26, 45, 48, 50, 54, 57). Although the most commonly infected cells appear to be multinucleated giant cells and macrophages or monocytes which express CD4 on their cell surface, HIV may also be found in occasional endothelial cells, astrocytes, oligodendrocytes, neurons, and glial cells. The role of CD4 in HIV infection of the nervous system has yet to be resolved. In this regard, several investigators have documented that HIV can infect both CD4⁺ and CD4⁻ continuous human neural cell lines as well as low-passage neural cell populations derived from human fetal brain specimens (6-8, 10, 12, 21). These infections, however, are very inefficient and result in only a small number of infected cells. It has been suggested that these cells may be only minimally permissive for HIV replication and may harbor the virus in a latent or persistent form. Unfortunately, it is difficult to separate restriction at the adsorption step from intracellular restriction to replication. With the use of HIV pseudotypes, one can begin to address issues relating specifically to the intracellular events.

Our data show that the HIV (Ampho-1B) pseudotypes can infect not only CD4⁻ human cells but also mouse cells. The different cell types, however, showed considerable variability with respect to the amount of virus produced, suggesting an important role for intracellular factors in modulating the HIV infection. Similar conclusions have been reached by others who studied HIV replication following transfection of infectious molecular clones of HIV into a variety of human and nonhuman CD4⁻ cells (1, 29). In our experiments, the HIV pseudotypes replicated to the highest titers in the human FF cells, and as monitored by in situ cytohybridization, all cells in the culture appeared to be infected. The temporal appearance of HIV-specific proteins in the HIV pseudotype-infected FF cells also was similar to that seen following infection of CD4⁺ CEM cells. The human glioblastoma cells and HeLa cells were somewhat less efficient hosts for the HIV pseudotypes than the FF cells, with approximately 7- to 10-fold-lower titers produced following infection. The least amount of virus was produced following infection of the mouse cells, although the BALB/c MEC seemed to be capable of sustaining persistent HIV infection longer than the wild mouse SC-1 cell lines. These differences

may be due, at least in part, to the slower growth of the primary cultures, which required fewer passages during the course of the experiment. Since the HIV pseudotypes are only phenotypically altered, the HIV pseudotype infection in the CD4⁻ cells is primarily restricted to a single round of replication, unless there is concomitant infection of the cell with the amphotropic retrovirus. Thus, infected cells containing unintegrated viral DNA or exhibiting a growth disadvantage would progressively become a smaller proportion of the total cell population in rapidly dividing cells. In addition, intracellular factors needed for efficient HIV gene expression or assembly into virions likely vary between the different cell types. It is clear, however, that HIV infection of these various cell types with the HIV pseudotypes is far more efficient than infections in which HIV or transfections with molecular clones of HIV are used. Furthermore, Chesebro et al. (7) have recently shown that even after introduction of a CD4 expression vector into some CD4⁻ human cell lines, the cells were still resistant to HIV infection, but this block to infection could be overcome by HIV virions pseudotyped with the envelope protein of the amphotropic retrovirus 4070A. Thus, these HIV pseudotypes will likely have great utility for studying HIV replication and pathogenesis in many different cell types and potentially in animal models. In addition, these pseudotypes may be useful for studies involving naturally occurring HIV variants defective in *env* (15).

An important issue raised by these studies is whether HIV pseudotypes can form in vivo with other enveloped viruses. The broad spectrum of clinical disease associated with HIV infection argues that the virus may have multiple options for expanding its ability to infect different types of cells, including both genomic heterogeneity and pseudotype formation. Although the amphotropic retrovirus is not a pathogen in patients with AIDS, these individuals are infected with multiple enveloped viruses. Furthermore, it has been documented that coinfection of brain cells by human cytomegalovirus and HIV does occur in these patients (42). Therefore, the possibility exists that in vivo, dual infection of a cell with HIV and an enveloped virus could yield pseudotypes, thus expanding the cellular and species tropism of the HIV progeny and potentially having an impact on the pathogenesis of AIDS.

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