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Pseudotyping with Human T-Cell Leukemia Virus Type I Broadens the Human Immunodeficiency Virus Host Range

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Several epidemiologic and clinical studies suggest that patients coinfected with human immunodeficiency virus (HIV), the primary etiologic agent in AIDS, and other viruses, such as cytomegalovirus or human T-cell leukemia virus (HTLV), have a more severe clinical course than those infected with HIV alone. Cells infected with two viruses can, in some cases, give rise to phenotypically mixed virions with altered or broadened cell tropism and could therefore account for some of these findings. Such pseudotypes could alter the course of disease by infecting more tissues than are normally infected by HIV. We show here that HIV type 1 (HIV-1) efficiently incorporates the HTLV type I (HTLV-I) envelope glycoprotein and that both HIV-1 and HTLV-II accept other widely divergent envelope glycoproteins to form infectious pseudotype viruses whose cellular tropisms and relative abilities to be transmitted by cell-free virions or by cell contact are determined by the heterologous envelope. We also show that the mechanism by which virions incorporate heterologous envelope glycoproteins is independent of the presence of the homologous glycoprotein or heterologous *gag* proteins. These results may have important implications for the mechanism of HIV pathogenesis.

A large proportion of human immunodeficiency virus (HIV)-infected individuals are also infected with human T-cell leukemia virus type I (HTLV-I) or HTLV-II (16), cytomegalovirus (8, 30), or human herpesvirus 6 (32). Several studies suggest that those infected with a second virus have a more aggressive form of the disease than those infected with HIV alone (2, 13a, 43, 46). It has been proposed that increased transcriptional activation of HIV by other viruses, such as herpesviruses (14, 22, 23), papovaviruses (11), hepatitis B virus (33), and HTLV (35), may account for these findings. It is possible that, in addition to cross-transactivation, phenotypic mixing of HIV with other viruses plays a role in pathogenesis.

Mixing of envelope glycoproteins in doubly infected cells to form pseudotype virions has been demonstrated for several of the enveloped viruses (reviewed in reference 1). Recently it was reported that HIV produced in cells infected with xenotropic murine leukemia virus (MLV) (18), amphotropic MLV (A-MLV) (6, 38), or herpes simplex virus (51) has an expanded host range, suggesting that pseudotyped virions had formed. There is suggestive evidence of a role for pseudotypes in pathogenesis in the feline system, in which it has been shown that cats coinfected with feline immunodeficiency virus (FIV) and feline leukemia virus have a more aggressive immunodeficiency than those infected with FIV alone (26). While the amount of feline leukemia virus present in the coinfected cats was not increased, FIV was found in nonlymphoid tissues, which are not normally targets of the virus.

The mechanism by which viruses incorporate homologous or heterologous envelope glycoproteins is poorly understood. Incorporation of the homologous envelope has been postulated to require interaction of the Gag matrix protein with the transmembrane domain of the envelope glycoprotein. This model is supported by the finding that $p19^{gag}$ of Rous sarcoma virus can be chemically cross-linked to $gp37^{env}$, indicating that the two proteins are in contact in the virion (10). It is not clear, however, that this interaction drives incorporation of envelope glycoprotein molecules into the virion. In Rous sarcoma virus, the cytoplasmic tail of the transmembrane protein is not essential for envelope incorporation, as truncation of this segment did not decrease the infectivity or the amount of mutant envelope glycoprotein in the virions (27).

Pseudotypes have previously been formed only in doubly infected cells that contain all the components of the two viruses. Thus, it has not been clear whether heterologous envelope glycoprotein incorporation depends on incorporation of the homologous envelope glycoprotein or, possibly, on incorporation of small amounts of the heterologous *gag* proteins. We have established methods for the generation of HIV type 1 (HIV-1) and HTLV-II pseudotype viruses that allowed us to test the ability of these viruses to incorporate heterologous envelope glycoproteins in the presence or absence of the homologous envelope glycoprotein and in the absence of other heterologous viral components. This approach also allowed us to study the role that the envelope glycoprotein plays in determining the host range and mode of transmission of HIV and HTLV.

MATERIALS AND METHODS

Cell culture. Cells were maintained in Dulbecco modified Eagle medium H21 containing 10% heat-inactivated fetal calf serum (GIBCO, Long Island, N.Y.). HeLa-T4 cells were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (donated by R. Axel).

Construction of *env* HIV and HTLV vectors. Construction of the HIV-1 vector, HIV-gpt, is described elsewhere (24). The HTLV-II vector, SV-HTLV-II-neo, was constructed by ligating the infectious HTLV-II clone H6.1 (34) into the simian virus 40 (SV40)-based expression vector pSV7d (40). A portion of the *env* gene, removed by cleaving with *Apa*I and *Hin*cII (positions 5542 to 6215), was replaced with a

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restriction fragment containing the SV40 origin of replication and early-region promoter-enhancer linked to the *neo* gene (37). The *env* deletion does not affect the *tax-rex* exons.

Construction of envelope glycoprotein expression vectors. The HIV envelope glycoprotein vector, SV-HIV-*env*, was constructed by ligating the *Sac*I (nucleotides 5999 to 8896; 39) fragment of HIV HXB2 to *Sma*I-digested pSV7d. The HTLV-I envelope glycoprotein vector, SV-HTLV-I-*env*, was constructed by ligating a *Hin*dIII-*Pst*I-cleaved full-length HTLV-I *env* cDNA to similarly cleaved pSV7d. The Rev-responsive element of HIV (*Bgl*II-*Hin*dIII, positions 7620 to 8140) was placed in the 3′ untranslated region of the *env* gene. A-MLV and ecotropic MLV (E-MLV) envelope glycoprotein vectors, SV-A-MLV-*env* and SV-E-MLV-*env*, were constructed by ligating the *Bgl*II-*Nhe*I fragment of Moloney MLV or A-MLV 4070a (nucleotides 5407 to 7846; 13) to *Bam*HI-*Xba*I-digested pSV7d. The MLV long terminal repeat (LTR) (*Eco*RI-linkered *Eco*RI-*Hin*dIII fragment of Ψ^- Moloney MLV [20]) was inserted at the *Eco*RI site between the SV40 enhancer-promoter and *env* genes of both plasmids.

Virus production. COS cells (1.2×10^6) were seeded in 10-cm-diameter dishes and transfected the next day with 15.0 μg of each plasmid plus, in some cases, 3.0 μg of pcRev by the calcium-phosphate-chloroquine procedure (42). The precipitate was replaced by fresh medium after 12 h, and the cells were refed 24 and 48 h later. Virus was harvested 72 h after transfection, filtered through a 0.45-μm-pore-size membrane, and used without freezing.

Cell-free infection. Virus produced by the transfected COS cells (0.5, 0.1, and 0.01 ml) was incubated in a total volume of 0.5 ml with 2.0×10^5 cells in a 6-well culture dish. After 2 to 4 h, 0.5 ml of medium was added. Virus was removed after 12 h, and 2.0 ml of medium was added to each well. Two days after infection, 1/20 of the cells was transferred to mycophenolic acid selection medium (Dulbecco modified Eagle medium containing 10% dialyzed fetal calf serum, 250 μg of xanthine per ml, 14 μg of hypoxanthine per ml, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0], and 50 μg of mycophenolic acid [Calbiochem-Behring, La Jolla, Calif.] per ml) or to neomycin selection medium (medium supplemented with 250 μg of G418 per ml). Cells were fed every 3 days, and after 10 to 12 days, colonies were stained with 0.2% crystal violet–25% isopropanol–5% acetic acid and counted. Colonies in the well containing the greatest number of discrete colonies were counted, normalized to 1.0 ml of virus, and multiplied by 5 to account for the 1:20 split and the approximately fourfold increase in cell number between the time of infection and selection.

Infection by cocultivation. COS cells were treated 48 h after transfection for 3 h with 10 μg of mitomycin C per ml. Treated COS cells (approximately 2×10^5 and 0.25×10^5 per well) were mixed with 2.0×10^5 target cells and seeded in a 6-well culture dish containing 2.0 ml of medium. After 2 days, half of the cells were transferred into selective medium. Colonies were counted in the well containing the greatest number of discrete colonies and presented as colonies per 2.5×10^5 transfected COS cells.

Immunoblot analysis of retroviral proteins. Plasmid DNA (30 μg of envelope expression vector with or without 3.0 μg of pcRev) was transfected into COS cells. Cell lysates were prepared 60 h later by removing cells from the tissue culture dish with calcium-magnesium-free phosphate-buffered saline containing 0.5 mM EDTA. The cells were lysed in buffer containing 1% Triton X-100, 10 mM Tris (pH 7.5), 1.5 M NaCl, and 2.0 mM EDTA. Nuclei were removed by centrifugation in an Eppendorf microcentrifuge for 10 min at 4°C. A volume of the lysate containing 200 μg of protein was denatured and reduced by heating to 90°C for 90 s in sample buffer containing 2-mercaptoethanol. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon filters (Millipore Corp., Bedford, Mass.) as previously described (42). The filters were blocked by treatment for 1 h with Tris (pH 8.0)–0.15 M NaCl–1% Tween 20–1% gelatin (TBST). The filter-bound proteins were treated with antibody and then with the appropriate alkaline phosphatase-conjugated anti-immunoglobulin antibody diluted 1:8,000 in TBST. Bound antibody was visualized by developing the filter in 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and Nitro Blue Tetrazolium chloride. The filters were probed with monoclonal anti-
gp120 antibody 110-1 (41) diluted 1:10,000, monoclonal anti-HTLV-I envelope glycoprotein antibody 1C11 diluted 1:1,000 (25), or goat anti-E-MLV-env serum diluted 1:10,000.

RESULTS

Use of recombinant vectors to produce pure retrovirus pseudotypes. To study the ability of HIV and HTLV to be pseudotyped by heterologous envelope glycoproteins, we developed *env* mutant retrovirus vectors in which a portion of the *env* gene was replaced by a selectable marker gene. In the HIV-1 vector, HIV-gpt, the *env* gene has been replaced by the SV40 early-region promoter linked to the *Escherichia coli* guanine phosphoribosyl transferase gene (construction and characterization of the HIV-gpt vector is described in reference 24). In the HTLV-II vector, the *env* gene was replaced by the neomycin resistance gene under control of the SV40 early-region promoter-enhancer (Fig. 1, SV-HTLV-II-neo). HTLV-II was used instead of HTLV-I because no infectious HTLV-I clone has been described. Cotransfection of COS cells with either of the retrovirus vectors and plasmids encoding various retrovirus envelope glycoproteins (Fig. 1) should result in release of replication-defective virions having different host ranges. Although expression of HIV and HTLV envelope glycoproteins requires coexpression of Rev or Rex, respectively, it is not necessary to supply expression vectors for these two regulatory proteins in the cotransfections, as these proteins are produced by the retrovirus vectors themselves.

Cell-cell and cell-free transmission of virus were assessed by cocultivating the transfected COS cells with various target cells or by mixing the filtered cell supernatant with target cells; the colonies appearing after growth in medium containing mycophenolic acid or G418 (for HIV-gpt- or SV-HTLV-II-neo-derived virus, respectively) were then counted.

This method of producing pseudotypes has several experimental advantages over methods in which pseudotypes are produced by infecting cells with two viruses. Alterations in host range of these pseudotypes can be attributed to the envelope glycoprotein and not to phenotypic mixing of other viral components. In addition, the effects of other interactions that occur between viruses in doubly infected cells, such as cross-transactivation, are eliminated. Pseudotypes produced in doubly infected cells contain a mixture of envelope glycoproteins, whereas viruses produced in this system contain a single envelope glycoprotein that mediates binding to and entry into target cells; the need to inhibit the

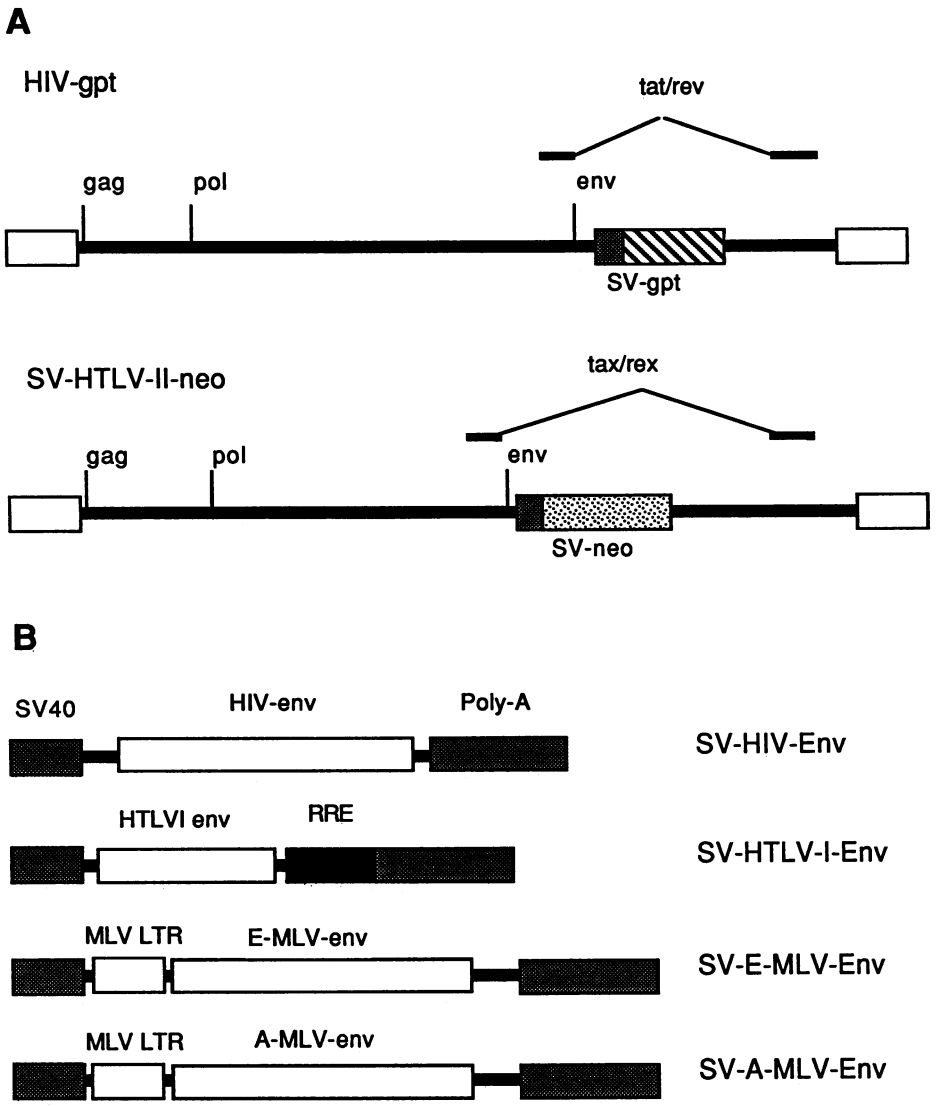

FIG. 1. Recombinant plasmids for production of HIV-1 and HTLV-II pseudotypes. (A) Recombinant HIV-1 and HTLV-II vectors, HIV-gpt and HTLV-II-neo. (B) Structure of the retrovirus *env* expression vectors. In plasmids SV-HIV-I-*env* and SV-HTLV-I-*env*, transcription is driven by the SV40 enhancer-promoter. In plasmids SV-A-MLV-*env* and SV-E-MLV-*env*, transcription is driven by the MLV LTR. In SV-HTLV-I-*env*, the Rev-responsive element (RRE) of HIV-1 was placed in the 3′ untranslated region of a full-length HTLV-I *env* cDNA clone to allow transport of the mRNA to the cytoplasm in the presence of Rev. SV-A-MLV-*env* and SV-E-MLV-*env* contain the SV40 enhancer-promoter, the LTR of Moloney MLV, and the A-MLV (strain 4070a; 13) or E-MLV *env* gene.

function of the homologous envelope by antiserum treatment is therefore eliminated.

Envelope glycoprotein expression vectors produce appropriately processed products. Recombinant plasmid vectors expressing the envelope glycoproteins of HIV-1, HTLV-I, and A-MLV or E-MLV were constructed and are shown in Fig. 1. Each vector contains the SV40 origin to allow for replication in COS cells. HIV-1 and HTLV-II *env* transcription is controlled by the SV40 promoter-enhancer. MLV *env* transcription is controlled by the MLV LTR (vectors in which MLV *env* transcription was driven by the SV40 promoter-enhancer resulted in an unsuitably low level of protein production [data not shown]).

The ability of the vectors to direct envelope glycoprotein synthesis was assessed by immunoblot analysis of COS cells transfected with SV-HIV-I-*env* or SV-HTLV-I-*env* with or without the Rev expression vector pcRev (19). In the absence of Rev, HIV-1 and HTLV-I envelope glycoproteins were expressed at low or undetectable levels, respectively (Fig. 2A, lane 3, and B, lane 1). In the presence of Rev, bands corresponding to the processed and precursor forms of the HIV-1 envelope glycoprotein (gp120 and gp160, Fig. 2A, lane 4; control infected cell lysate, lane 1) and of the HTLV-I envelope glycoprotein (gp46 and faint heterogeneous band between 61 and 66 kDa; Fig. 2B, lane 2) were observed. Fluorescence-activated cell sorter analysis showed that both envelope glycoproteins were present at the cell surface, although the level of gp46 was considerably lower than that of gp160 (data not shown). Lysates of COS cells transfected with SV-E-MLV-*env* and SV-A-MLV-*env* contained bands corresponding to the precursor and processed forms of the MLV envelope glycoprotein gp70 (Fig. 2C, lanes 3 and 5). These

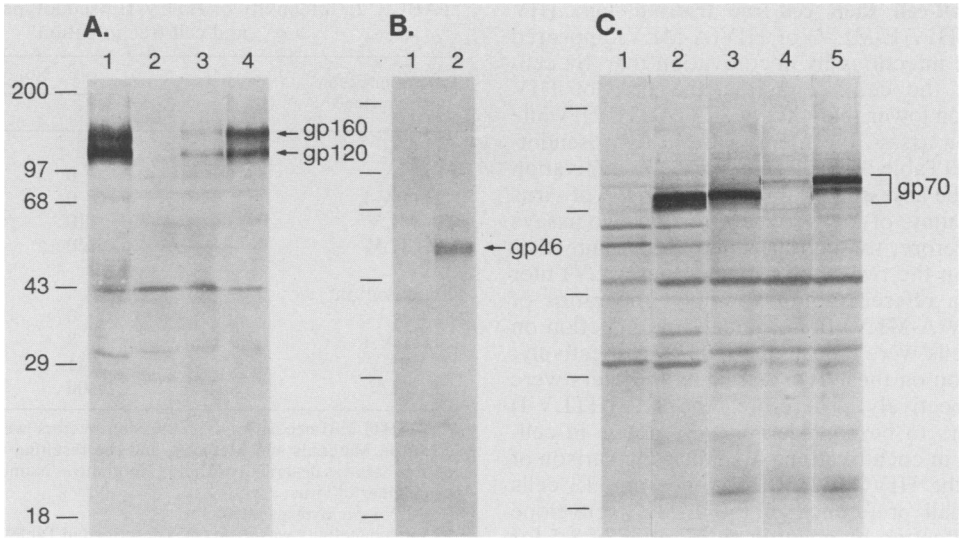

FIG. 2. Immunoblot analysis of envelope glycoproteins expressed by COS cells transfected with expression vectors. (A) Hut78 cells infected with HIV (lane 1) and COS cells transfected with pcRev (lane 2), SV-HIV-I-*env* (lane 3), and SV-HIV-I-*env* and pcRev (lane 4) and probed with monoclonal anti-gp120 antibody 110-1 (41). (B) COS cells transfected with SV-HTLV-I-*env* (lane 1) and pcRev and SV-HTLV-I-*env* (lane 2) and probed with monoclonal anti-HTLV-I envelope glycoprotein antibody 1C11 (25). (C) 3T3 cells stably transfected with E-MLV provirus deleted in the *env* gene (lane 1) or with E-MLV provirus (lane 2); COS cells transfected with SV-E-MLV-*env* (lane 3), A-MLV provirus (lane 4), or SV-A-MLV-*env* (lane 5) probed with goat anti-E-MLV-envelope glycoprotein serum. This serum has some reactivity with MLV *gag* proteins that are visible in lanes 1 and 2. Numbers at the left are sizes in kilodaltons.

products comigrated with the envelope glycoproteins from control cells, producing wild-type E- or A-MLV (Fig. 2C, lanes 2 and 4).

HIV(MLV) and HIV(HTLV) pseudotypes have altered cell tropisms and host ranges. To test the ability of HIV-1 to form pseudotypes with heterologous envelope glycoproteins, COS cells were cotransfected with the envelope expression vectors and the HIV-1 vector HIV-gpt. The resulting viruses were tested for infectivity on human and murine cell lines using cell-free and cocultivation protocols (as described in Materials and Methods). Virus produced by COS cells transfected with HIV-gpt in the absence of envelope glycoprotein showed an undetectable (NIH 3T3 and HeLa) or a very low (HOS and HeLa-T4) level of infection in cell-free and cocultivation assays (Table 1). Virus produced by COS cells cotransfected with HIV-gpt and the gp160 vector SV-HIV-I-*env* displayed the expected host range for HIV, efficiently infecting only $CD4^+$ human cells (HeLa-T4) by either cell-free or cocultivation routes (Table 1). Virus produced by COS cells cotransfected with HIV-gpt and A-MLV or E-MLV *env* vectors had the host range characteristic not of HIV, but of the virus from which the envelope glycoprotein was derived: HIV(E-MLV) infected only murine cells (3T3), and HIV(A-MLV) infected both human and murine cells (HeLa, HeLa-T4, HOS, and 3T3). These pseudotypes were infectious by cell-free infection and cocultivation (Table 1). HIV pseudotyped by the HTLV-I envelope glycoprotein infected $CD4^-$ and $CD4^+$ human cells and inefficiently infected 3T3 cells, which is consistent with previous reports of low levels of HTLV receptor on murine cells (36). Interestingly, the titer of HIV(A-MLV) in cell-free infection was considerably higher on HeLa-T4 cells than that of HIV bearing the homologous envelope glycoprotein gp160. This difference in titer is probably not due to a difference in expression level of the two envelope glycoproteins, because by immunoblotting, the protein levels appear similar (Fig. 2). While this interpretation assumes similar sensitivity of the two antibodies in detecting envelope glycoprotein, it is supported by the finding that two other antibodies gave similar results (data not shown). The significance of this finding is discussed below.

Pseudotypes bearing the HTLV-I envelope glycoprotein are

TABLE 1. Infectivity of HIV pseudotypes by cocultivation and cell-free infection

Type of infection and envelope	No. of colonies on:			
	3T3	HOS	HeLa	HeLa-T4
Cell free[a]				
—[b]	0	5	0	5
HIV-1	0	50	0	1,800
HTLV-I	0	175	80	NT[c]
A-MLV	5,600	173,500	25,000	14,995
E-MLV	47,800	0	0	0
Cocultivation[d]				
—[b]	0	0	0	0
A-MLV	295	800	408	75
E-MLV	11,100	0	0	0
HIV-1	0	8	0	588
HTLV-I	3	980	524	139
E-MLV and HIV-1	5,600	0	NT	660
HTLV-I and HIV-1	0	932	226	498

[a] HIV-gpt and *env* expression vectors were cotransfected as described in Materials and Methods. Medium was harvested 72 h after transfection. Virus (0.5, 0.1, and 0.01 ml) was incubated with 2×10^5 cells, and 2 days later, 1/20 of the cells was transferred to mycophenolic acid selection medium. Cell free infection was measured per milliliter of virus.

[b] —, No *env* expression vector.

[c] NT, Not tested.

[d] Transfected COS cells were treated after 48 h with 10 μg of mitomycin C per ml for 3 h and washed twice with phosphate-buffered saline. Cells (approximately 2.5×10^5 and 0.25×10^5 per well) were seeded together with 2×10^5 target cells. After 2 days of cocultivation, half of the cells were transferred into selective medium. Control cultures in which mitomycin C-treated COS cells were incubated without target cells showed no colonies (data not shown). Each experiment was performed at least three times with similar results. Cocultivation was measured per 2×10^5 COS cells.

more active in cell-cell than cell-free transmission. HIV (HTLV-I), unlike HIV(E-MLV) or HIV(A-MLV) appeared considerably more infectious by cocultivation than by cell-free infection. In the cell-free assay, the titer of HIV (HTLV-I) was much lower than that of HIV(A-MLV), while in the cocultivation assay, the titers of these two pseudotypes were similar (Table 1). Cell-free versus cocultivation infectivity cannot be assessed by direct comparison of virus titers because the units of measurement for the two assays are different. Therefore, to quantitate the relative infectivities of each virus in the two assays, the HIV(A-MLV) titer can be taken as a reference. For example, the ratios of HIV(HTLV-I)/HIV(A-MLV) titers by cell-free infection on HOS and HeLa cells were 0.0010 and 0.0032, respectively, and by cocultivation on these two cell lines, the titers were 1.2 and 1.3, respectively. Therefore, the HIV(HTLV-I) pseudotype appears to be considerably less active in cell-free infection than in cocultivation. A similar comparison of HIV(E-MLV) to the HIV(A-MLV) reference on 3T3 cells reveals only a small preference of the E-MLV envelope glycoprotein for infection by cocultivation (ratios of 8.5 for cell-free infection compared with 38.0 for cocultivation). By this analysis, the HIV-1 envelope glycoprotein shows a somewhat larger preference for cocultivation than E-MLV glycoprotein, but this preference is considerably lower than that of the HTLV-I envelope glycoprotein [(HIV(HIV)/HIV(A-MLV) ratios on HeLa-T4 cells of 0.12 for cell-free infection compared with 7.8 for cocultivation].

Efficiency of pseudotyping is unaffected by the presence of the homologous envelope glycoprotein. Because HIV pseudotypes produced by this method are assembled in the absence of gp160, they may not be representative of those formed in doubly infected cells. It is conceivable that gp160 affects the frequency of pseudotyping, either negatively (by competing with heterologous envelope protein for interaction sites on the capsid) or positively (by providing nucleation sites for the aggregation of viral components at the cell membrane, as has been postulated previously [49]). To examine these possibilities, COS cells were cotransfected with a mixture of HIV-gpt, SV-HIV-I-*env*, and either SV-E-MLV-*env* or SV-HTLV-I-*env*, and the viruses produced were assayed by cocultivation. Both HIV(E-MLV) and HIV(HTLV-I) pseudotype formation appeared unaffected by the presence of the HIV-1 envelope glycoprotein (Table 1). In addition, in the case of the E-MLV-*env*–HIV-I-*env* mixture, the titer on HeLa-T4 was similar to that of HIV bearing the HIV-1 envelope glycoprotein alone. Therefore, it can be concluded that the HIV-1 envelope glycoprotein was not detectably inhibited from being incorporated into the virion by the presence of the heterologous envelope glycoprotein. It is unclear, however, whether these virions consisted of a single population containing both envelope glycoproteins or two populations, each with a single envelope glycoprotein. However, pseudotypes produced in other systems have been shown to consist of mixed envelope-containing virions (44, 45, 49).

Pseudotyping of HTLV permits more efficient cell-free transmission. Previous reports have noted the inability of HTLV-I and -II to be transmitted by cell-free virus preparations (5, 50). Cell-free transmission was achieved in one study, but this could have been a very low-efficiency event (7). The observation that HIV(HTLV-I) pseudotypes were more efficiently transmitted via cocultivation than as cell-free virions suggested that the HTLV envelope glycoprotein may be responsible for this preference. To test this hypothesis, we prepared reciprocal pseudotypes using the HTLV-II retrovirus vector. SV-HTLV-II-neo was cotransfected with the HTLV-I E-MLV and A-MLV envelope glycoprotein vectors, and the resulting viruses were tested by cocultivation and cell-free infection of human and murine cells (Table 2). The titers produced with the HTLV-II vector were considerably lower than those produced with the HIV vector. This may be because of the previously described weak transcriptional activity of the HTLV LTR in nonlymphoid cells such as the COS cells used in our work (4). As expected, cotransfection of the HTLV-I *env* vector with SV-HTLV-II-neo resulted in virus that infected HOS cells by cocultivation but not by cell-free infection. In contrast, cotransfection of A- or E-MLV *env* vectors resulted in virus that readily infected cells by cell-free infection and cocultivation and showed the appropriate host range [HOS and 3T3 for HTLV(A-MLV) and 3T3 for HTLV(E-MLV)]. From these results we conclude that HTLV-II can be pseudotyped by MLV envelope glycoproteins and that the low infectivity of cell-free HTLV-II virions can be overcome by exchanging the glycoprotein.

TABLE 2. Infectivity of HTLV-II pseudotypes by cocultivation and cell-free infection

Type of infection and envelope	No. of colonies on:	
	3T3	HOS
Cell free[a]		
—[b]	0	0
HTLV-I	0	0
A-MLV	110	245
E-MLV	220	0
Cocultivation[c]		
—[b]	0	0
HTLV-I	8	88
A-MLV	141	154
E-MLV	1,300	0

[a] SV-HTLV-II-neo and *env* expression vectors were transfected as described in Materials and Methods, and the resulting virus was assayed by cell-free assay as described in Table 1, footnote *a*. Number of colonies is given per milliliter of virus.

[b] —, No *env* expression vector.

[c] Virus infectivity was assayed as described in Table 1, footnote *d*. Number of colonies is given per 2×10^5 COS cells.

DISCUSSION

The results of this study demonstrate that HIV-1 can be readily pseudotyped by the envelope glycoprotein of HTLV-I, resulting in expanded cell tropism and host range. In addition, HIV-1 can be pseudotyped by the E-MLV envelope glycoprotein and, in agreement with recent reports (38), by the A-MLV envelope glycoprotein. Similarly, we show here that HTLV-II can be pseudotyped by the A- and E-MLV envelope glycoproteins. Each pseudotype displayed the tropism characteristic of the heterologous envelope glycoprotein it had incorporated. These pseudotypes differ from those recently reported and from those produced by double infections in other viral systems in that these were produced in the absence of the homologous envelope glycoprotein and therefore contain pure rather than mixed envelopes. This fact enabled us to make several observations about the mechanisms by which pseudotypes are formed.

The mechanisms that control the incorporation of envelope glycoproteins into virions are not well understood. Envelope glycoproteins appear to be specifically incorpo-

rated, since nearly all other cell membrane proteins are excluded from the viral envelope (3, 17, 31). It has been suggested, in the case of rhabdovirus pseudotypes, that heterologous envelope glycoproteins are incorporated into the assembling virion by associating on the cell surface with homologous envelope glycoprotein molecules. The homologous envelope glycoprotein could then serve to nucleate the budding reaction (49). In those pseudotypes, the heterologous envelope glycoprotein does not substitute for the homologous glycoprotein but instead is believed to occupy interstices between the homologous envelope glycoprotein molecules (21). In that case, the ability of the heterologous envelope glycoprotein to be incorporated into the virion could be due to its ability to associate with the homologous glycoprotein. In contrast, the pseudotypes produced in our experiments were formed in the absence of the homologous envelope glycoprotein. Furthermore, the presence of the homologous envelope glycoprotein failed to enhance pseudotype formation. Our results are more consistent with a model of envelope glycoprotein incorporation in which some feature common to many envelope glycoproteins, such as a protein structural motif or the ability to form a two-dimensional array of envelope glycoprotein molecules on the cell membrane, is recognized by the assembling core particle. An alternative model that cannot be ruled out by our results is that envelope and core components may be targeted to a specific region of the cell membrane from which most cellular proteins are excluded, thereby allowing the formation of virions containing only the virion-encoded components. This model seems unlikely, however, because virions appear to bud at random positions from the cell surface.

While it is thought that phenotypic mixing does not involve extensive mixing of virion proteins other than the envelope glycoprotein (44, 45), it is possible that pseudotyping requires the incorporation of small amounts of heterologous Gag that serves to stimulate the incorporation of heterologous envelope glycoprotein molecules into the virion. Our results indicate that this is unlikely to be the case, because heterologous Gag was absent during the assembly of these pseudotypes.

The titers of HIV(MLV) were higher than those of HIV-1 bearing the homologous envelope glycoprotein, suggesting that the envelope glycoproteins of MLV function more effectively than gp160. This could be the result of the inefficient processing and transport to the cell surface that has been observed for gp160, the majority of which is targeted to lysosomes for degradation (48). In addition, because gp120 is not covalently linked to gp41, it may be easily removed from virions. Thus, pseudotyping of HIV-1 can increase its infectivity as well as broaden its host range.

Similarly, pseudotyping of HTLV-II increases the infectivity of the virions produced. In this case, the incorporation of a MLV envelope glycoprotein considerably increased the infectivity of cell-free virions. One explanation for these results is that the HTLV-I envelope glycoprotein is unstable and largely inactive in cell-free preparations of virus. Cocultivation would allow the virus to spread more efficiently because it is exposed to target cells shortly after budding. Pseudotyping in an infected individual could give rise to virions capable of being transmitted without the need for cell-cell contact. Such particles might therefore infect tissues not normally accessible to the virus.

In the course of these studies we have developed vectors useful for the study of HTLV. HTLV has been difficult to study because of its slow growth and poor transmissibility. By using SV-HTLV-II-neo, it is possible to rapidly produce virus and quantitate its infectivity (but not its replication, because of its *env* deletion). In addition, the biohazard associated with these virions is expected to be much lower than that of replication-competent virus.

Pseudotyping has not yet been demonstrated in individuals infected with HIV and another virus. However, it is likely that doubly infected cells are present in individuals infected with HIV and HTLV, since both viruses are lymphotropic. Viruses produced by such cells would be capable of infecting tissues not normally infectable with HIV. Phenotypic mixing of HIV with HTLV, cytomegalovirus, Epstein-Barr virus, human herpesvirus 6, or other viruses frequently found in HIV-infected individuals could account for the spread of the virus to $CD4^-$ cells. HIV can be found in the $CD4^+$ macrophages of the brain but has also been reported to be present in $CD4^-$ astrocytic, neuronal, and endothelial cells (9, 29, 47). Infection of cultured cells of neural lineage has been reported to occur by a CD4-independent mechanism (12, 15) that could involve recognition by gp160 of a non-CD4 cell surface molecule or could be due to the presence of HIV pseudotypes arising in cells producing a second virus. Such pseudotypes may have an important role in the pathogenesis of HIV-related syndromes, such as those involving the central nervous system.

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REFERENCES

1. **Boettiger, D.** 1979. Animal virus pseudotypes. Prog. Med. Virol. **25**:37–68.
2. **Bonetti, A., R. Weber, M. W. Vogt, W. Wunderli, W. Siegenthaler, and R. Luthy.** 1989. Co-infection with human immunodeficiency virus-type-1 (HIV-1) and cytomegalovirus in two intravenous drug users. Ann. Intern. Med. **111**:293–296.
3. **Calafat, J., H. Janssen, P. Demant, J. Hilgers, and J. Zavada.** 1983. Specific selection of host cell glycoproteins during assembly of murine leukaemia virus and vesicular stomatitis virus: presence of thy-1 glycoprotein and absence of H-2, Pgp-1 and T-200 glycoproteins on the envelopes of these virus particles. J. Gen. Virol. **64**:1241–1253.
4. **Chen, I. S. Y., J. McLaughlin, and D. W. Golde.** 1984. Long terminal repeat of human T-cell leukaemia virus II genome determine target cell specificity. Nature (London) **309**:276–279.
5. **Chen, I. S. Y., S. G. Quan, and D. W. Golde.** 1983. Human T-cell leukemia virus type II transforms normal human lymphocytes. Proc. Natl. Acad. Sci. USA **80**:7006–7009.
6. **Chesebro, B., R. Buller, J. Portis, and K. Wehrly.** 1990. Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J. Virol. **64**:215–221.
7. **Clapham, P., K. Nagy, R. Cheingsong-Popov, M. Exley, and R. A. Weiss.** 1983. Productive infection and cell-free transmission of human T-cell leukemia virus in a nonlymphoid cell line. Science **222**:1125–1127.
8. **Fiala, M., L. A. Cone, C. Chang, and E. Mocarski.** 1986. Cytomegalovirus viremia increases with progressive immune deficiency in patients infected with HTLV-III. AIDS Res. **2**:175–181.

9. **Gabuzda, D. H., D. D. Ho, S. M. de la Monte, M. S. Hirsch, T. R. Rota, and R. A. Sobel.** 1986. Immunohistochemical identification of HTLV-III antigen in brains of patients with AIDS. Ann. Neurol. **20**:289–295.
10. **Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis.** 1984. Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes. An insight into virus assembly. J. Mol. Biol. **174**:297–317.
11. **Gendelman, H. E., W. Phelps, L. Feigenbaum, J. M. Ostrove, A. Adachi, P. M. Howley, G. Khoury, H. S. Ginsberg, and M. A. Martin.** 1983. Transactivation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83**:9759–9763.
12. **Harouse, J. M., C. Kunsch, H. T. Hartle, M. A. Laughlin, J. A. Hoxie, B. Wigdahl, and F. Gonzalez-Scarano.** 1989. CD4-independent infection of human neural cells by human immunodeficiency virus type 1. J. Virol. **63**:2527–2533.
13. **Hartley, J. W., and W. P. Rowe.** 1976. Naturally occurring murine leukemia viruses in wild mice: characterization of a new amphotropic class. J. Virol. **19**:19–25.

13a. **Hattori, T., A. Koito, K. Takatsuki, S. Ikematsu, J. Matsuda, H. Mori, M. Fukui, K. Akashi, and K. Matsumoto.** 1989. Frequent infection with human T-cell lymphotropic virus type I in patients with AIDS but not in carriers of human immunodeficiency virus type 1. J. Acquired Immune Defic. Syndr. **2**:272–276.

14. **Horvat, R. T., S. Wood, and N. Balachandran.** 1989. Transactivation of human immunodeficiency virus promoter by human herpesvirus 6. J. Virol. **63**:970–973.
15. **Kunsch, C., H. T. Hartle, and B. Wigdahl.** 1989. Infection of human fetal dorsal root ganglion glial cells with human immunodeficiency virus type 1 involves an entry mechanism independent of the CD4 T4A epitope. J. Virol. **63**:5054–5061.
16. **Lee, H., P. Swanson, V. S. Shorty, J. A. Zack, J. D. Rosenblatt, and I. S. Chen.** 1989. High rate of HTLV-II infection in seropositive I.V. drug abusers in New Orleans. Science **244**:471–475.
17. **Lodish, H. F., and M. Porter.** 1980. Specific incorporation of host cell surface proteins into budding vesicular stomatitis virus particles. Cell **19**:161–169.
18. **Lusso, P., F. di Marzo Veronese, B. Ensoli, G. Franchini, C. Jemma, S. E. DeRocco, V. S. Kalyanaraman, and R. C. Gallo.** 1990. Expanded HIV-1 cellular tropism by phenotypic mixing with murine endogenous retroviruses. Science **247**:848–852.
19. **Malim, M. H., J. Hauber, R. Fenrick, and B. R. Cullen.** 1988. Immunodeficiency virus rev trans-activator modulates the expression of the viral regulatory genes. Nature (London) **335**:181–183.
20. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
21. **Metsikko, K., and H. J. Garoff.** 1989. Role of heterologous and homologous glycoproteins in phenotypic mixing between Sendai virus and vesicular stomatitis virus. J. Virol. **63**:5111–5118.
22. **Mosca, J. D., D. P. Bednarik, N. B. Raj, C. A. Rosen, J. G. Sodroski, W. A. Haseltine, and P. M. Pitha.** 1987. Herpes simplex virus type-1 can reactivate transcription of latent human immunodeficiency virus. Nature (London) **325**:67–70.
23. **Ostrove, J. M., J. Leonard, K. E. Weck, A. B. Rabson, and H. E. Gendelman.** 1987. Activation of the human immunodeficiency virus by herpes simplex virus type 1. Virology **12**:3726–3732.
24. **Page, K. A., N. R. Landau, and D. R. Littman.** 1990. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J. Virol. **64**:5270–5276.
25. **Palker, T. J., M. E. Tanner, R. M. Scearce, R. D. Streilein, M. E. Clark, and B. F. Haynes.** 1989. Mapping of immunogenic regions of human T cell leukemia virus type I (HTLV-I) gp46 and gp21 envelope glycoproteins with env-encoded synthetic peptides and a monoclonal antibody to gp46. J. Immunol. **142**:971–978.
26. **Pedersen, N. C., M. Torten, B. Rideout, E. Sparger, T. Tonachini, P. A. Luciw, C. Ackley, N. Levy, and J. Yamamoto.** 1990. Feline leukemia virus infection as a potentiating cofactor for the primary and secondary stages of experimentally induced feline immunodeficiency virus infection. J. Virol. **64**:598–606.
27. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61**:2981–2988.
28. **Price, R. W., B. Brew, J. Sidtis, M. Rosenblum, A. C. Schneck, and P. Cleary.** 1988. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. Science **239**: 5586–5591.
29. **Pumarola-Sune, T., B. A. Navia, C. Cordon-Cardo, E. S. Cho, and R. W. Price.** 1987. HIV antigen in the brains of patients with the AIDS dementia complex. Ann. Neurol. **21**:490–496.
30. **Raffi, F., D. Boudart, and S. Billaudel.** 1990. Acute co-infection with human immunodeficiency virus (HIV) and cytomegalovirus. Ann. Intern. Med. **112**:234–235.
31. **Russ, G., K. Polakova, and J. Zavada.** 1983. Assembly of xenotropic murine leukaemia virus related antigens from the surface of mouse L cells by vesicular stomatitis virus. Acta Virol. **27**:105–109.
32. **Salahuddin, S. Z., D. V. Ablashi, P. D. Markham, S. F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R. C. Gallo.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science **234**:596–601.
33. **Seto, E., Y. Benedict, T. S. Yen, B. M. Peterlin, and J. Ou.** 1988. Transactivation of the human immunodeficiency virus long terminal repeat by the hepatitis B virus X protein. Proc. Natl. Acad. Sci. USA **85**:8286–8290.
34. **Shimotohno, K., W. Wachsman, T. Takahashi, D. W. Golde, M. Miwa, T. Sugimura, and I. S. Y. Chen.** 1984. Nucleotide sequence of the 3′ region of an infectious human T-cell leukemia virus type II genome. Proc. Natl. Acad. Sci. USA **83**:6657–6661.
35. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science **238**:1575–1578.
36. **Sommerfelt, M. A., B. P. Williams, P. R. Clapham, E. Solomon, P. N. Goodfellow, and R. Weiss.** 1988. Human T cell leukemia viruses use a receptor determined by human chromosome 17. Science **242**:1557–1559.
37. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
38. **Spector, D. H., E. Wade, D. A. Wright, V. Koval, C. Clark, D. Joquish, and S. A. Spector.** 1990. Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. J. Virol. **64**:2298–2308.
39. **Starcich, B., L. Ratner, S. F. Josephs, T. Okamoto, R. C. Gallo, F. Wong-Staal.** 1985. Characterization of long terminal repeat sequences of HTLV-III. Science **227**:538–540.
40. **Stuve, L. L., S. Brown-Shimer, C. Pachl, R. Najarian, D. Dina, and R. L. Burke.** 1987. Structure and expression of the herpes simplex virus type 2 glycoprotein gB gene. J. Virol. **61**:326–335.
41. **Thomas, E. K., J. N. Weber, J. McClure, P. R. Clapham, M. C. Singhal, M. K. Shriver, and R. A. Weiss.** 1988. Neutralizing monoclonal antibodies to the AIDS virus. AIDS **2**:25–29.
42. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60**:755–765.
43. **Webster, A., D. G. Cook, V. C. Emery, C. A. Lee, J. E. Grundy, P. B. A. Kernoff, and P. D. Griffiths.** 1989. Cytomegalovirus infection and progression towards AIDS in haemophiliacs with human immunodeficiency virus infection. Lancet **ii**:63–66.
44. **Weiss, R. A., and P. L. P. Bennett.** 1980. Assembly of membrane glycoproteins studied by phenotypic mixing between mutants of vesicular stomatitis virus and retroviruses. Virology **100**:252–274.

45. **Weiss, R. A., and A. L. Wong.** 1977. Phenotypic mixing between avian and mammalian RNA tumor viruses. I. Envelope pseudotypes of Rous sarcoma virus. Virology **45**:508–515.
46. **Wiley, C. A., and J. A. Nelson.** 1988. Role of human immunodeficiency virus and cytomegalovirus in AIDS encephalitis. Am. J. Pathol. **133**:73–81.
47. **Wiley, C. A., R. D. Schrier, J. A. Nelson, P. W. Lampert, and M. B. A. Oldstone.** 1986. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proc. Natl. Acad. Sci. USA **83**:7089–7093.
48. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85**:9580–9584.
49. **Witte, O. N., and D. Baltimore.** 1977. Mechanism of formation of pseudotypes between vesicular stomatitis virus and murine leukemia virus. Cell **11**:505–511.
50. **Yamamoto, N., M. Okada, Y. Koyanagi, M. Kannagi, and Y. Hinuma.** 1982. Transformation of human leukocytes by cocultivation with an adult T cell leukemia virus producer cell line. Science **217**:737–739.
51. **Zhu, Z., S. S. L. Chen, and A. S. Huang.** 1990. Phenotypic mixing between human immunodeficiency virus and vesicular stomatitis virus or herpes simplex virus. J. Acquired Immune Defic. Syndr. **3**:215–219.