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Differential Expression in Human and Mouse Cells of Human Immunodeficiency Virus Pseudotyped by Murine Retroviruses

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Expression of cell surface CD4 influences susceptibility of cells to human immunodeficiency virus (HIV) infection; however, some CD4-positive human and mouse cells are still resistant to HIV infection. To search for mechanisms of resistance to HIV independent of CD4 expression, HIV expression was studied in human and mouse cells normally resistant to HIV infection by introducing infectious virus by transfection of HIV DNA or infection with HIV pseudotyped with amphotropic or polytropic murine leukemia viruses. The results indicated that even when barriers to viral entry were bypassed, mouse NIH 3T3 cells and Dunni cells still showed a marked reduction in number of cells expressing HIV compared with the human cells studied, although the intensity of immunostaining of individual positive mouse cells was indistinguishable from that seen on permissive human cell lines. CD4 expression in mouse cells or human brain or skin cells did not influence the number of HIV foci observed after transfection with HIV DNA or infection with pseudotyped HIV. These results suggested that in addition to a block in the usual HIV fusion and entry process, CD4-positive mouse cells differed from human cells in exhibiting partial resistance to HIV infection which acted at a postpenetration step in the infection cycle. This resistance was partially overcome when mouse cells were infected by direct exposure to human lymphocytes producing HIV pseudotyped by amphotropic murine leukemia virus.

CD4 is believed to be a major cell surface receptor for human immunodeficiency virus (HIV). However, in acquired immunodeficiency syndrome patients, not all CD4-positive cells are infected by HIV. Furthermore, recent studies indicate that not all CD4-positive human cell lines can be efficiently infected in vitro by HIV (3). CD4 expressed on certain astroglial and skin tumor cell lines specifically bound HIV, but HIV envelope protein was unable to mediate fusion with the plasma membranes of these cells. Thus, the virus could not enter and infect the cells efficiently. This block in cell penetration by HIV was overcome when HIV genomes were contained in pseudotyped particles with amphotropic murine leukemia virus (MuLV) envelope protein [HIV(Ampho)], which could then enter the cells by using the receptor for amphotropic MuLV (3). After infection by HIV(Ampho), there was no apparent block in postpenetration steps of HIV infection in these human cell lines.

In CD4-positive mouse cell lines, HIV has similarly been found to bind to CD4 but does not fuse the cells or undergo successful infection (15). However, after transfection with infectious HIV DNA, CD4-positive or -negative mouse cells produced detectable infectious HIV (1). These results suggested that the barriers to successful HIV infection in mouse cells might be mainly at the point of virus entry. However, expression of infectious HIV in mouse cells has not been easy to quantitate after transfection, and it remains possible that in addition to a block of virus entry, there are other regulatory effects which decrease HIV expression in these cell lines. Furthermore, since many mouse cell lines are highly susceptible to infection by amphotropic MuLV, HIV(Ampho) would also be expected to infect these cells unless they have mechanisms of resistance to HIV which act at steps after virus entry. In order to compare mechanisms of resistance to HIV in human and mouse cell lines, HIV expression was quantitatively compared after introducing HIV by infection with virus particles with amphotropic MuLV envelopes or by transfection with infectious HIV DNA from a molecularly cloned plasmid.

Comparison of infection of mouse and human cells by HIV and HIV pseudotyped with amphotropic MuLV. Our previous results indicated that HIV pseudotyped with amphotropic MuLV envelope protein [HIV(Ampho)] was capable of productive infection in certain nonpermissive human cell lines independent of CD4 expression (3). HIV(Ampho) was produced by establishing a chronic infection of amphotropic MuLV in human A3.01 leukemia cells and then superinfecting these cells with HIV. In addition to the input virus types, such dually infected A3.01 cells should also release pseudotyped virions in which one viral RNA was contained in a particle where the predominant envelope protein was derived from the coinfecting retrovirus (19, 20). Thus, HIV(Ampho) stocks should consist of a mixture of pseudotyped particles with either HIV or amphotropic MuLV RNA as well as unpseudotyped particles of each virus. In order to see whether HIV(Ampho) was also capable of infecting mouse cells, we did quantitative assays comparing infection by HIV alone with infection by HIV(Ampho).

In these experiments, two different HIV strains, LAV and NL4-3, were also compared. Results indicated that efficient infection of HeLa cells by LAV and NL4-3 correlated with CD4 expression (Table 1). However, in the other human and mouse cell lines tested, there was little if any infection even in those lines which expressed CD4. In contrast, amphotropic pseudotypes of both LAV and NL4-3 were capable of infecting all the cell lines tested; however, there were large quantitative differences observed in the number of foci detected (Table 1). Whereas LAV(Ampho) infected all the human cell lines at approximately equal levels, the titer was reduced by over 100-fold on most mouse cell lines tested. NL4-3(Ampho) differed somewhat in that it showed a higher level of infectivity in the three mouse cell lines than was seen

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TABLE 1. HIV infection of CD4-negative and CD4-positive human and mouse cell lines by pseudotyped HIV[a]

Species	Cell line	CD4	No. of HIV foci/0.2 ml				No. of HIV infectious centers/10^6 cells			
			LAV	LAV(Ampho)	NL4-3	NL4-3(Ampho)	LAV	LAV(Ampho)	NL4-3	NL4(Ampho)
Human	HeLa clone 1022	+	3900	6,600	5,880	16,000	28,000	38,000	26,000	41,000
	HeLa	−	0	1,580	0	2,000	20	19,300	6	18,800
	U87 clone 2	+	0	7,240	1	34,000	530	11,000	290	51,000
	U87	−	0	3,240	0	36,000	1	16,000	2	44,000
	SCL1 clone 6	+	1	3,160	1	6,300	6	11,000	14	20,000
	SCL1	−	0	3,880	0	16,600	2	10,500	7	52,000
Mouse	NIH clone 5	+	0	10	0	446	95	2,600	2	11,700
	NIH 3T3	−	0	22	0	512	1	4,950	1	11,000
	Dunni	−	0	60	0	850	11	3,000	8	6,200

[a] All assays were done in 24-well trays seeded with 3×10^4 cells the day before infection. By day 3 after infection, each well contained about 3×10^5 cells. Values shown are geometric means of replicate dilution titrations; standard errors were ≤40% of values shown. Except in the cases of HeLa and clone 1022 cells infected by cell-free pseudotyped HIV, the differences between pairs of CD4-positive and CD4-negative cells in titers of pseudotyped HIV were not statistically significant. HIV pseudotyped with amphotropic MuLV (4070A) was produced in A3.01 lymphocytes chronically infected with amphotropic MuLV as described previously (3). These cells were then superinfected with HIV, and 5 to 7 days later, when HIV infection was extensive, supernatant fluid was collected for a cell-free virus stock, or A3.01 cells were used as infectious centers. CD4-positive mouse NIH 3T3 cells (clone 5) were generated by methods described before (3); other target cells were described previously (3, 12; B. Chesebro, K. Wehrly, J. Metcalf, and D. E. Griffin, J. Infect. Dis., in press). In separate experiments, human HeLa, U87, and SCL1 cells were found to be 50-, 10-, and 33-fold less infectible, respectively, by amphotropic MuLV than mouse NIH 3T3 and Dunni cells (data not shown).

with LAV(Ampho), but titers on human cells were still much higher than on mouse cells. In separate experiments with amphotropic MuLV alone, mouse NIH 3T3 and Dunni cells gave 10- to 50-fold higher titers than the three human cell lines (data not shown). Thus, the mouse cells expressed a functional receptor for amphotropic MuLV envelope protein; nevertheless, there appeared to be a marked quantitative reduction in the number of HIV foci induced by HIV(Ampho) in mouse cells relative to human cells. These results suggested that there were postpenetration restrictions on HIV expression in these mouse cells compared with human cells.

Since a higher multiplicity of infection can be delivered when infected cells are cocultivated on susceptible target cells, retroviral infection is often much more efficient when virus is transmitted by cell-to-cell contact (6, 17). Therefore, we also tested HIV expression in various mouse and human target cells after cocultivation with A3.01 human leukemia cells producing HIV or HIV(Ampho). Results indicated that infected A3.01 cells producing only HIV NL4-3 or LAV were highly efficient at infecting CD4-positive HeLa cells but were markedly reduced in their effectiveness in all the other cell lines (Table 1). There was a small but significant effect of CD4 expression in the U87 clone, where the number of foci detected was increased in CD4-positive cells with NL4-3; however, this higher level was still 100- to 500-fold lower than that seen in susceptible CD4-positive HeLa cells. In contrast, A3.01 cells producing pseudotyped HIV(Ampho) particles were highly effective at infecting all the cells, and the mouse and human cells differed by only about fourfold with this technique (Table 1). Thus, it appeared that the number of hits delivered by either the HIV or the amphotropic MuLV could contribute to partially overcoming the postpenetration resistance to HIV infection exhibited by these mouse cell lines, and in this respect this phenomenon appeared to be quite similar to the effect of the *Fv-1* gene on MuLV cell tropism (7). This effect could be due either to the multiplicity of HIV genomes delivered to each infected cell or to a "helper" effect secondary to a dual infection by both amphotropic MuLV and HIV.

Specificity of interference by infection with HIV(MuLV) pseudotypes. In order to test the receptor specificity of HIV pseudotyped with MuLV, we used HeLa cells infected with various MuLV strains to see whether preinfection could interfere with subsequent infection by HIV(MuLV) pseudotypes. In these experiments we also produced HIV pseudotypes by using another MuLV, polytropic Friend MCF virus, F-MCF-98D (2, 4) [HIV(98D)], to compare the effectiveness and specificity of this virus with previous studies with amphotropic MuLV. The results in Tables 2 and 3 showed that interference occurred and was specific for the particular HIV pseudotype used to challenge the target cells. Thus, HIV(Ampho) was unable to infect HeLa cells previously infected with amphotropic MuLV but was successful at infecting HeLa cells preinfected with F-MCF-98D. Similar results were obtained when either cell-free virus stocks (Table 2) or infected A3.01 human lymphocytes (Table 3) were used to transfer the infection. In contrast, HIV pseudotyped with F-MCF-98D could only be detected when infected lymphocytes were tested (Table 3). The level of HIV(98D) detected was very low compared with that seen with HIV(Ampho). However, the predicted interference

TABLE 2. Specificity of interference with cell-free pseudotyped HIV[a]

Target cells		No. of HIV foci/0.2 ml			
Cells	Interfering virus	HIV	HIV (Ampho)	HIV (98D)	HIV (Mol-Ampho)
HeLa-CD4	None	7,320	50,000	6,130	22,900
HeLa	None	0	4,170	0	4,680
HeLa	Ampho	0	0	0	0
HeLa	98D	0	13,500	0	11,200

[a] HIV pseudotyped with F-MCF-98D, a polytropic MuLV [HIV(98D)], was produced by HIV infection of A3.01 human leukemia cells previously chronically infected with F-MCF-98D (2, 4). HIV pseudotyped with Moloney MuLV containing the amphotropic MuLV envelope gene [HIV(Mol-Ampho)] was produced by HIV infection of CD4-positive HeLa clone 6C cells (5). These cells were infected with the Moloney-amphotropic recombinant MuLV derived from PA12 cells at the time of transfer of the CD4 retroviral expression vector. HIV(Mol-Ampho) was then propagated by infection of A3.01 cells. Target cells for these interference assays by the focus induction technique (5) were CD4-positive HeLa cell clone 1022, which does not contain Moloney or amphotropic MuLV envelope sequences or any MuLV capable of replication on mouse NIH 3T3 cells (Chesebro et al., in press), normal HeLa cells, and HeLa cells previously infected with amphotropic MuLV (4070A) or F-MCF-98D. Values shown are geometric means of replicate titrations, and standard errors were ≤40% of values shown.

TABLE 3. Specificity of interference with pseudotyped HIV produced by dually infected human lymphocytes[a]

Target cells		No. of HIV foci/10^6 lymphocytes			
Cells	Interfering virus	HIV	HIV (Ampho)	HIV (98D)	HIV (Mol-Ampho)
HeLa-CD4	None	29,500	66,100	24,600	53,700
HeLa	None	0	23,000	65	33,100
HeLa	Ampho	0	1	38	0
HeLa	98D	0	34,700	0	21,400

[a] See Table 2, footnote *a*.

specificity was observed, as infection by HIV(98D) was blocked in HeLa cells infected with F-MCF-98D but not in cells infected with amphotropic MuLV. These results showed that, similar to the case with mouse cells (4), F-MCF-98D and amphotropic MuLV used different receptors on HeLa cells, and in cells chronically infected with one or the other MuLV, these receptors were unavailable for use by the corresponding pseudotyped HIV. The reason for the low level of detection of HIV pseudotyping by F-MCF-98D is not known. However, in human HeLa, U87, and SCL1 cells, F-MCF-98D is 40- to 400-fold less infectious than amphotropic MuLV (data not shown). Therefore, it is possible that the efficiency of detection of HIV(98D) pseudotypes might be quite low in these human cells due to poor infectivity of the pseudotyping MuLV. Alternatively, there might be some restriction in the association of HIV RNA with the core and/or envelope proteins of F-MCF-98D, which could also explain the low titers observed for HIV(98D) pseudotypes.

We also tested HIV pseudotyped with a recombinant MuLV consisting of amphotropic envelope sequences inserted in the genome of Moloney MuLV [HIV(Mol-Ampho)]. This virus was derived from our previously described CD4-positive HeLa cell clone 6C (5) and was the result of the rescue of the murine retroviral packaging gene sequences present in PA12 cells (16) used originally for generation and transfer of the CD4 gene to HeLa cells. We have subsequently found that 6C cells produce replication-competent MuLV capable of infection of NIH 3T3 cells (data not shown), and analysis of HIV produced by these cells indicated that a substantial portion of this virus was capable of infecting CD4-negative HeLa cells (Tables 2 and 3). This pseudotyped HIV(Mol-Ampho) appeared to use the amphotropic envelope to penetrate HeLa cells, since infection did not occur in HeLa cells preinfected with amphotropic MuLV (Tables 2 and 3). Thus, HIV passaged in 6C cells had a receptor specificity similar to that of HIV(Ampho) obtained from mixed infection of A3.01 human lymphocytes.

HIV expression after transfection of mouse and human cell lines. In order to eliminate the steps of virus penetration and uncoating which might influence infection by HIV(Ampho), we also introduced HIV genomes into human and mouse cells by transfection of DNA from an infectious HIV molecular clone (1). Plasmid pNL4-3 DNA (5 μg) was used to transfect CD4-positive and CD4-negative mouse and human cell lines by the calcium phosphate procedure. Cells were split 1:4 the next day and were monitored for the number of HIV-positive cells by direct immunostaining and for production of infectious HIV by cocultivation with A3.01 cells (Table 4). Results indicated that all cells produced infectious HIV, although virus was detectable earlier from human cells than from mouse cells. There was a marked quantitative difference among the various cell lines in the number of cells expressing HIV, but CD4 expression appeared to have no influence on the number of HIV foci detected following transfection. Among the human cells, HeLa cells had the most HIV-positive cells (>3%), followed by U87 astroglioma cells (0.7%) and then by SCL1 squamous cell carcinoma cells (0.046%). HIV-positive cells were also observed with the three mouse cell lines tested, but the number detected was extremely low (0.002 to 0.006%). These results

TABLE 4. HIV expression, CAT activity, and neomycin resistance after transfection of human and mouse cell lines[a]

Species	Cell line	CD4	Infectious HIV	HIV-positive cells/ 5×10^5 cells	Neo^r colonies/ 5×10^5 cells	CAT activity[b] (%)
Human	HeLa clone 1022	+	++	>16,000	NT[c]	NT
	HeLa	−	++	>16,000	520 ± 15	30.4
	U87 clone 2	+	++	3,568 ± 482	NT	NT
	U87	−	++	3,488 ± 176	100 ± 10	51.3
	SCL1 clone 6	+	++	222 ± 34	NT	NT
	SCL1	−	++	236 ± 20	20 ± 7	18.2
Mouse	NIH clone 5	+	+	10 ± 2	NT	NT
	NIH 3T3	−	+	16 ± 4	585 ± 15	0.44
	Dunni	−	+	30 ± 2	967 ± 203	0.13

[a] Cell lines used were described previously (3; Chesebro et al., in press). Cells (5×10^5) in the 60-mm dish were transfected with 5 μg of plasmid DNA by the $CaPO_4$ procedure. pLSNL7 (18) containing the neomycin resistance gene was used as a standard for transfection efficiency, and data are expressed as the mean ± SE of neomycin (G418)-resistant colonies per 5×10^5 cells transfected as observed at 14 days posttransfection. Cells transfected with pNL4-3 containing an infectious molecular clone of HIV (1) were split 1:4 24 h after transfection, and on days 2, 3, and 5 dishes were scored directly for HIV-expressing cells per 5×10^5 original transfected cells by the immunoperoxidase method. Data shown are the mean ± SE of results on days 2 and 3. To test for production of infectious HIV, cells were cocultivated for 24 h with A3.01 human CD4-positive lymphocytes at 3 days posttransfection. A3.01 cells were then moved to a separate dish for continued growth, and cells were monitored for HIV expression by reverse transcriptase assay and focal immunoassay on CD4-positive HeLa cells (5). HIV was detected by focal immunoassay in A3.01 cells at 3 (++) or 7 (+) days after cocultivation.

[b] CAT assays were performed as described by Gorman et al. (9). Five micrograms of the plasmids pUCBennCAT and pAR (8) were transfected into the human and mouse cell lines by the $CaPO_4$ method. pUCBennCAT contains the HIV LTR ligated to the bacterial gene for CAT (*cat*), and pAR contains an HIV LTR-*tat* construct that expresses Tat. The transfected cells were lysed at 48 h posttransfection, and cell lysates were analyzed for their ability to form acetylated [^{14}C]chloramphenicol. The acetylated and unacetylated products were separated by thin-layer chromatography, and CAT activity was quantitated by liquid scintillation counting of the acetylated and unacetylated forms of chloramphenicol. The β-galactosidase-expressing plasmid pCH110 (10) was cotransfected with the *cat* constructs. The CAT activity represents percent acetylation of [^{14}C]chloramphenicol per standard lysate volume. Lysates were diluted to obtain percent acetylation values in the linear range (0.1 to 25%) and were normalized to β-galactosidase activity to correct for transfection efficiency. Data presented are the average of several experiments.

[c] NT, Not tested.

were similar to those observed after infection of mouse cells by HIV(Ampho) and supported the conclusion that there was a significant postpenetration restriction of HIV expression in mouse cells.

Transfection efficiency was controlled by parallel transfection with a murine retroviral vector expressing the neomycin resistance gene by using the Moloney MuLV long terminal repeat (LTR) as a promoter (18). The mouse cells and HeLa cells had similar high levels of neomycin-resistant colonies, and U87 and SCL1 cells had 5- and 25-fold fewer, respectively. Thus, the differences in HIV expression in mouse cells versus HeLa cells did not appear to be due to transfection efficiency, but instead supported the conclusion based on infection with pseudotyped HIV that there was a significant postpenetration resistance of HIV expression in mouse cells.

In order to measure more directly the function of HIV promoter and transactivation activities in these cell lines, cotransfections were done with HIV LTR-*cat* and HIV LTR-*tat* plasmids (Table 4). The results indicated that chloramphenicol acetyltransferase (CAT) activity in both mouse cell types was greatly reduced compared with all three human cell lines. This suggested that the low number of HIV-positive cells in transfected mouse cells might be due to poor HIV LTR function. Recent results with hamster-human cell hybrids indicate that high HIV expression correlated with the presence of human chromosome 12 (11). Presumably, the absence of genes on human chromosome 12 might also account for low HIV expression in mouse cells. The mechanism of this effect remains unclear. However, by measuring HIV protein expression with immunoperoxidase staining in transfected cell cultures, we observed that those HIV-positive mouse cells which were present appeared to stain nearly as intensely as did human HeLa cells. Thus, the main difference observed between HeLa cells and mouse cells was in the frequency of HIV-positive cells seen rather than the level of protein expressed in each positive cell. Similarly, the decreased CAT activity observed in transfected mouse cells might also represent normal transcription levels in a very reduced number of cells rather than an overall reduced level of transcription in many cells.

The results of CAT experiments were also helpful in interpreting the HIV expression data in the human cell lines. Interestingly, CAT activity was highest in U87 cells. Thus, the lower number of HIV-positive cells after transfection of U87 cells was probably due to inefficiency of transfection, as demonstrated by the lower number of neomycin-resistant colonies induced by a control plasmid. The higher activity of the HIV LTR in U87 cells also agrees with the higher numbers of HIV foci after HIV(Ampho) infection (Table 1), since in this case the delivery of the HIV genome to U87 cells was done through a more efficient infection step. In contrast, SCL1 cells were somewhat lower in CAT activity than HeLa or U87 cells. SCL1 also had the lowest transfection efficiency. Together, these findings could explain the low numbers of HIV-positive cells seen after HIV transfection of SCL1 cells (Table 4).

The results of these studies indicate that restriction of HIV infection can occur at several different levels. Virus entry can be blocked by lack of CD4 expression, as in HeLa cells. Alternatively, in the presence of CD4, virus entry can be blocked by a lack of successful virus-cell fusion, as in CD4-positive U87 and SCL1 human cells (3) and in CD4-positive mouse NIH 3T3 cells (15). In contrast, infection can also be partially restricted at some step after viral penetration and uncoating, perhaps by regulation of transcription, as seen in several cell types (Table 4) (13). These different restrictions also can occur concurrently in a single cell type, and similar restrictions may account for some of the tissue and species tropism observed for HIV in vivo.

The phenomenon of HIV pseudotyping circumvents restrictions at the level of virus entry. Past and present results indicate that pseudotyping of HIV is not restricted to only one type of MuLV. HIV pseudotyping has now been demonstrated with amphotropic MuLV (3), F-MCF-98D (Tables 2 and 3), and a xenotropic MuLV derived from nude mice (14). Therefore, it seems likely that other retroviruses from other species, including humans, will also form HIV pseudotypes, and this could markedly alter the species specificity and pathogenicity patterns observed during infection.

In contrast to the results of Lusso et al. (14), we have not observed an increase in the rate of spread of HIV in MuLV-infected human A3.01 cells or HeLa cells. It is unclear how such an increase in HIV spread might occur, since chronic MuLV infection of human cells should interfere with the spread of pseudotyped HIV, as shown in Tables 2 and 3, and unpseudotyped HIV should be the only virus form capable of spread in human cell lines preinfected by MuLV. Using a monoclonal antibody reactive with amphotropic MuLV envelope protein, we noted that after infection with HIV(Ampho) pseudotypes, amphotropic MuLV spread faster than HIV (data not shown). By day 3, each focus of amphotropic MuLV infection involved greater than 100 cells, whereas each HIV focus involved only 2 to 8 cells. Thus, the higher level of MuLV expression and spread in these cells appeared to be capable of blocking spread of pseudotyped HIV at each focus, and the small number of HIV-infected cells in each focus might represent clonal outgrowth from a single originally infected cell rather than cell-to-cell virus spread in the focus.

The results of studies on pseudotyped HIV may have some relevance to the attempts to create useful animal models of HIV infection. It is possible that when SCID mice carrying human tissue are infected with HIV, the viruses form HIV pseudotypes by using endogenous MuLV present in these mice. This might in turn allow HIV spread from human to mouse cells in these animals, which would greatly complicate the disease process in this model. Alternatively, normal mice with no human cells might be susceptible to infection by HIV(Ampho) or other HIV(MuLV) pseudotypes, and this could provide a new animal model system. Our data showing that mouse cell lines have a significant restriction to infection by cell-free HIV(Ampho) suggest that the spread of pseudotyped HIV might also be restricted in vivo. However, transfer of HIV(Ampho) infection from infected human lymphocytes to mouse cells appeared to overcome this restriction (Table 1). Since cell-to-cell spread of virus may be the most important mode of infection in vivo, it is possible that HIV pseudotypes might spread quite well once established in mice.

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