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Factors Affecting Cellular Tropism of Human Immunodeficiency Virus

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To evaluate the basis of the slow growth of many human immunodeficiency virus strains in monocytes/macrophages, various stages of the virus life cycle have been studied for their possible contribution to viral tropism. Although we found that monocytic U937 cells had a higher percentage of CD4-positive cells than T-lymphoid H9 cells, the human immunodeficiency virus strain grew much less efficiently in the monocytic line. Viral tropism was primarily determined during the early stages of the virus cycle, that is, sometime between binding of the virus to the cell surface and reverse transcription of viral genomic RNA. Once the virus entered the host cell, reverse transcription, use of the long terminal repeat, RNA expression, and production of virus particles was about as efficient in monocytes as in T cells. Thus, during viral entry into the host cell cytoplasm there is a major limiting event that is particularly inefficient in U937 cells and possibly in all monocytes/macrophages.

The major cell types thought to be targets for human immunodeficiency virus (HIV) are T lymphocytes and monocytes/macrophages. Both display surface CD4 molecule, the known HIV receptor protein, and both are thought to be cellular reservoirs for HIV in infected people (for recent reviews, see references 10 and 15; 35). Certain strains of HIV grow well in T cells but poorly in monocytes/macrophages, while others exhibit the opposite growth pattern (9, 14). Understanding such viral tropism is vital to unraveling the pathogenetic mechanisms of HIV.

The retroviral life cycle consists of the processes of viral entry, reverse transcription of the RNA genome, integration of viral DNA, transcription, translation, posttranslational modification including protein processing, and assembly of the virus particle. Formally, the efficiency of viral growth, and eventually viral tropism, can be determined at any stage of the life cycle. For example, the main receptor of HIV is CD4 on the cell surface (7, 28), and thus its levels can influence the efficiency of viral infection; susceptibility to HIV infection generally, but not always, correlates with CD4 expression in host cells (1, 3). It has also been shown that intracellular levels of deoxyribonucleotides, the substrates for reverse transcription, can vary between host cells (19), suggesting that the rate of reverse transcription can be a factor affecting viral growth if multiple superinfection occurs. Complex interaction between viral and cellular factors is more evident at the level of gene expression, thereby affecting viral growth and tropism. The HIV long terminal repeat (LTR) interacts with a number of cellular factors whose expression is also modulated (13, 21, 31; for a recent review, see reference 24). The interplay of the various regulators, both viral and cellular, generates early and late transcriptional phases in the HIV life cycle; the earliest RNA is enriched in subgenomic species, while the genomic transcript appears at the later stage of infection (22). It is also possible that viral regulatory proteins, such as Tat and Rev, interact with cellular factors, because the magnitude of *trans*-activation of the HIV LTR by Tat varies between cell types and species (2, 6, 27, 29).

We have studied factors affecting viral tropism by analyzing the major stages of the virus life cycle during infection of different cell types. We have chosen well-known T-lymphocyte and monocyte cell lines which are CD4-positive but support very different rates of viral growth. Surprisingly, we found that slow viral growth in monocytic cells is largely due to inefficient viral entry, despite their high levels of surface CD4, and that all other steps of the virus life cycle appear to be as efficient in monocytic cells as in T cells.

MATERIALS AND METHODS

Cells, virus, and infection. Host cells for HIV infection were H9 (34) and U937 (37), T-lymphoid and monocytic cell lines, respectively. The levels of CD4 on the cell surface of these cells were analyzed by a fluorescence-activated cell sorter as described by Crowe et al. (5) using monoclonal antibody Leu3a (Becton Dickinson) against CD4 and Leu2a (Becton Dickinson) recognizing CD8 as a control monoclonal antibody. The viral strain used throughout this study was HIV-1-WI3 (22), which grows preferentially in T cells. Viral production was initiated by transfecting the plasmid pWI3 into H9 or COS cells. A high-titer preparation of viral stock was made by the shaking method as described previously (22, 39). Host cells were exposed to virus at 37°C for 1.5 h. They were then washed with medium and divided among an appropriate number of culture flasks. When H9 and U937 cells were compared for their infection by HIV, the same number of H9 and U937 cells were infected with equal quantities of virus by dividing freshly prepared virus precisely into two aliquots. At appropriate time points, samples were taken for reverse transcriptase assay (33), indirect immunofluorescence assay (20), viable-cell count, DNA, and RNA hybridization analysis.

Plasmids and transfection. The HIV LTR-CAT plasmid was constructed by ligating the *Kpn*I-*Hin*dIII fragment of HIV type 1 LTR into the *Pvu*II-to-*Hin*dIII sites of pSV918. pSV918 is identical to pSVECAT (16), except that the pBR322 backbone was replaced by SP65 (30). In the plasmid

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pSV-TAT, the *tat* coding region is driven by the simian virus 40 early promoter (8). Transfection of H9 and U937 cells was performed by the DEAE-dextran technique (36), as modified by Grosschedl and Baltimore (18) with 2 mg of HIV LTR-CAT and 4 mg of pSV-TAT plasmids per 10^7 cells. Chloramphenicol acetyltransferase (CAT) assays were performed by the procedure of Gorman et al. (16) with 20 to 30 mg of protein for each determination. The percentage of chloramphenicol that was acetylated was determined as described by Pierce et al. (32). To assess transfection efficiency, cells were transfected with pWI3 (22) which contains the whole viral genome. Forty-eight hours after transfection, the percentage of transfected cells was determined by measuring immunofluorescence-positive cells using human sera.

DNA and RNA blot hybridization. Preparation of the total DNA fraction (including low- and high-molecular-weight DNA), RNA, and the probe used for DNA hybridization analysis were as described by Kim et al. (22). The probe used for RNA hybridization analysis was antisense RNA, complementary to the 511-bp *Bgl*II fragment of the HIV LTR, which includes the polyadenylation signal sequence.

RESULTS

Inefficient viral growth in U937. The viral strain used in this study was HIV-1-WI3 (22). This strain was derived from one of several molecular clones from HIV-1_{IIIB} (34) and has been characterized previously (22, 23). The WI3 strain grows much less efficiently in monocytic cell lines than in T-lymphoid cell lines. In a particular infection (Fig. 1), HIV rapidly spread through the H9 culture soon after initial infection. The time point showing the highest percentage of infected cells (assayed by indirect immunofluorescence using human sera) or the highest reverse transcriptase activity in cell culture supernatants was obtained within 6 to 8 days after infection in H9 cells. In contrast, virus grew very slowly for the first 10 days in U937 cells, but more rapidly thereafter. Still, it took nearly 30 days to reach the peak of infection in U937 cells. Eventually, HIV infected 90 to 95% of cells in both cell types, and it was reproducibly found that the peak reverse transcriptase activity was higher in U937 cells than in H9 cells. Changes in the titer of the viral inoculum used for initial infection changed the length of the lag phase in the two cell types, but not the striking difference in the time required for the virus to reach the infection peak.

CD4 is not a limiting factor. To test whether surface CD4 concentration was a limiting factor for viral growth in U937 cells, we measured CD4 on the cell surface by fluorescence-activated cell sorter analysis. The apparent percentage of CD4-positive cells (recognized by Leu3a) was 43 and 100% in H9 and U937 cells, respectively (Fig. 2). The mean value for the level of CD4 in U937 cells was 1.6 times higher than in H9 cells. These results indicated that CD4 on the cell surface was not a limiting factor for viral growth in U937 cells.

LTR is used efficiently. There are many lines of evidence that the LTR of HIV plays a major role in viral gene expression (13, 21, 31; for a recent review, see reference 24). Therefore, using a transient transfection assay, we compared the degree of expression driven by the HIV LTR in U937 and H9 cells. An HIV LTR-CAT construct was transfected into each cell type with or without a *tat* expression vector (8), and transient expression of the bacterial CAT was used to compare the activities of the HIV LTR. For quantitative comparison, the same number of cells and the same quantity of plasmids were used in the transfection.

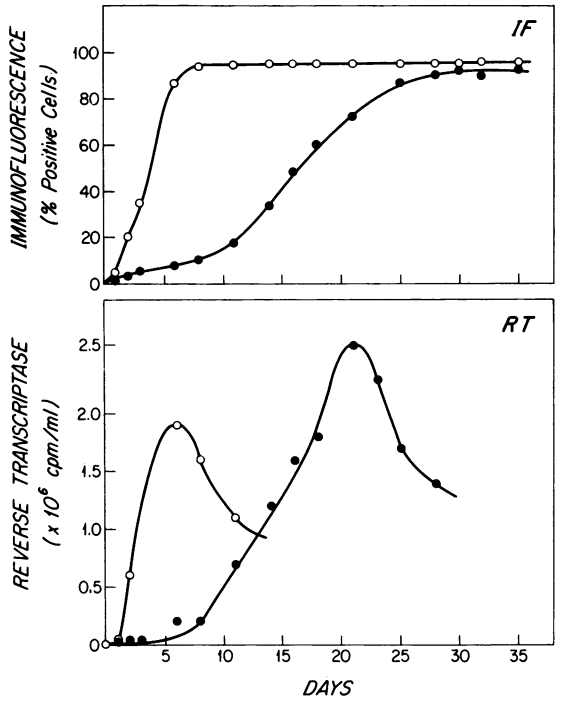

FIG. 1. Growth of HIV in H9 (○) and U937 (●) cells. Change in the percentage of immunofluorescence-positive cells (IF) using human sera or change in reverse transcriptase activity (RT) in cell culture supernatant. Note that a high titer of viral stock was used for the infection shown here. Under these conditions, we could synchronously infect 5 to 10% of H9 cells but only 0.5 to 1% of U937 cells. A single-cycle growth condition is achieved during the first 24 to 32 h postinfection (22). When 1% of the same viral stock was used, it took approximately 2 weeks for H9 cells and 2 to 3 months for U937 cells to reach the infection peak.

The percentages of transfected cells, as measured by immunofluorescence at 48 h after transfection, were 2 to 4% in both cell types, indicating that the transfection efficiencies in H9 and U937 cells were comparable. In both cell types, high levels of CAT activity were readily detectable when the HIV LTR-CAT plasmid was cotransfected with a *tat* expression vector (Fig. 3). Although the magnitude of difference in the LTR activity between the two cell types varied among the different laboratory clones of U937 used for transfection, the

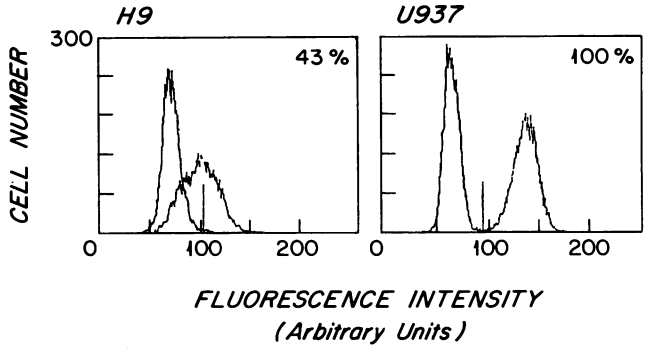

FIG. 2. Fluorescence-activated cell sorter analysis of CD4. Monoclonal antibody Leu3a (Becton Dickinson) against CD4 was used, while Leu2a (Becton Dickinson) recognizing CD8 was used as a control monoclonal antibody.

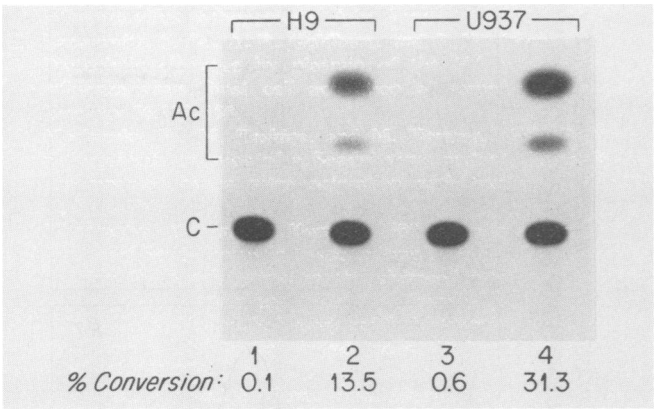

FIG. 3. CAT analysis of the HIV LTR. Lanes: 1 and 3, HIV LTR-CAT; 2 and 4, HIV LTR-CAT plus pSV-TAT. The result shown in this figure represents only one of many independent transfections. The transfection efficiency ranged from 2 to 4% in different transfections; for a given transfection, the transfection efficiencies in H9 and U937 cells were always comparable. C, Chloramphenicol; Ac, acetylated chloramphenicol.

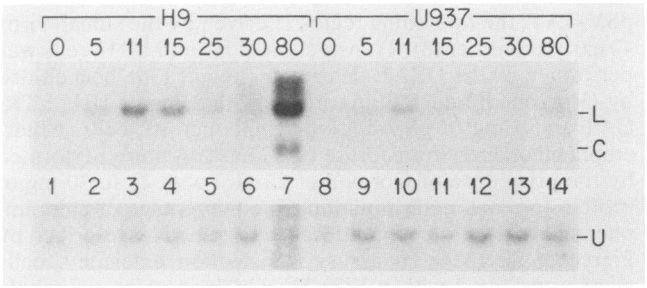

FIG. 5. Analysis of unintegrated HIV DNA. Numbers above the lanes are hours postinfection, while those between two panels indicate lane numbers. L and C are linear and circular DNA, respectively. U indicates the ~2.3-kb DNA bands used as DNA loading controls. This is a yet uncharacterized cellular genomic DNA which hybridized with our DNA probe (8) after *Xba*I digestion of cellular DNA samples. This band was present in all human cell lines and primary cells tested thus far. At present, it is not clear what this DNA band represents. The intensities of DNA bands were compared by laser densitometry (UltroScan laser densitometer LKB2202).

level of the CAT activity was always higher in U937 than in H9 cells. This result was consistent with our observation that the peak reverse transcriptase activity (Fig. 1) and RNA level per infected cell (data not shown) were also somewhat higher in U937 cells. This data suggests that the HIV LTR region may not be an apparent determinant of viral tropism.

Differential control of RNA expression appears identical. HIV shows a complex transcription pattern. We have previously shown that during HIV infection of T cells, there is a temporal progression of RNA accumulation, with the 2-kb subgenomic mRNA species being greatly enriched during the early transcriptional phase (22). The *rev* gene has been proposed to determine such differential RNA expression (22, 40). To investigate whether monocytes also show the same pattern of RNA expression, total RNAs were prepared from infected U937 cells at various time points before and after a one-step growth cycle and assayed for viral RNA content by electrophoretic separation and hybridization to a ^{32}P-labeled RNA probe (Fig. 4). During acute and chronic infection with HIV, three RNA bands were normally seen by RNA blot analysis (Fig. 4): the 9.2-kb band, which is both the genomic RNA and the mRNA for the *gag* and *pol* genes; the 4.3-kb species which includes *env* mRNA, as well as others, such as those for the partially spliced *tat* message; and the 2-kb mRNAs which encode the small regulatory genes such as *tat*, *rev*, and *nef*. The ratio of radioactivity in the 2-, 4.3-, and 9.2-kb mRNAs in long-term infected cells is approximately 1 to 0.9 to 2.5. When RNAs from infected U937 cells were analyzed, the first species, evident at 16 h postinfection, was the 2-kb RNA, with a small quantity of the 4.3-kb RNAs (Fig. 4). This corresponded to the pattern of early RNA expression previously seen in T cells (22). Therefore, HIV RNA expression in monocytes appears to be controlled in the same sequential manner as in T cells, suggesting that differential control of RNA expression may not be a major factor determining viral tropism.

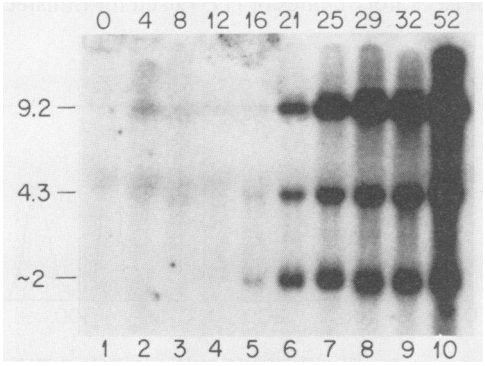

FIG. 4. Hybridization analysis of HIV RNA from a single-cycle infection culture. Numbers above the lanes are hours postinfection, while lane numbers are indicated at the bottom of HIV RNA panels. Numbers next to the panel indicate approximate sizes of RNA bands (in kilobases). Antisense RNA complementary to the 511-bp *Bgl*II fragment of pWI3 (8) was used as a probe.

Viral tropism is determined during the early stages of the virus life cycle. The above analyses suggested that the poor growth of our HIV strain in U937 cells was not determined at the level of gene expression. Therefore, we tested the possible role of earlier events in the virus life cycle in determining tropism. First, we measured the amount of unintegrated HIV DNA shortly after infection in H9 and U937 cells. This analysis determines the efficiencies of the processes of viral entry and reverse transcription. Total DNAs, including low- and high-molecular-weight DNA, were prepared at various time points after the initial infection. We have previously described the detailed kinetics of HIV DNA synthesis in T cells (8). The qualitative change of DNA synthesis during a single-step growth cycle was found to be essentially identical in both H9 and U937 cells; the quantities of viral DNA gradually increased until 11 h after infection and then decreased (Fig. 5). However, there was a significant difference between the two cell types in the quantity of linear DNA present, which is the major component of unintegrated DNA. The quantity of HIV linear DNA was reproducibly 5 to 10 times higher in H9 cells than in U937 cells (Fig. 5). This indicates that a major factor causing slow viral growth in U937 cells is in the early stages of the HIV life cycle, which includes the processes of viral entry and reverse transcription.

Viral entry is a major factor for determining viral growth. To distinguish between the involvement of reverse transcription and viral entry in viral tropism, we measured the

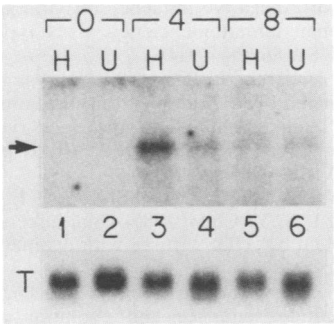

FIG. 6. Analysis of cell-associated viral genomic RNA. Numbers above lanes are hours postinfection, while numbers between panels indicate lane numbers. H and U are H9 and U937 cells, respectively. The viral genomic RNA is indicated as an arrow. T, α-tubulin RNA used as RNA loading controls (9, 26).

amounts of viral genomic RNA soon after infection of each cell type. For quantitative comparison, the same number of H9 and U937 cells were infected with equal amounts of virus, and cell-free virus was removed by washing infected cells with medium. Total RNAs were prepared and analyzed by RNA blot analysis. This analysis measures the amount of virion genomic RNA associated with host cells, and thus distinguishes the involvement of viral entry (including both viral binding and penetration) and reverse transcription in viral tropism. At 4 h after infection, the quantity of cell-associated virion RNA was reproducibly 5- to 10-fold higher in H9 cells than in U937 cells (Fig. 6). These results suggest that a major limiting factor(s) for viral growth in U937 occurs before the process of reverse transcription starts, probably during the process of viral entry.

DISCUSSION

We have studied factors affecting the efficiency of HIV infection in the monocytic cell line U937 as a model system for understanding viral tropism. Our results suggest that slow viral growth in U937 cells is due to inefficiency in the early stages of the virus life cycle, that is, sometime between binding of the virus to the cell surface and reverse transcription of viral genomic RNA. Once the virus enters the host cell, reverse transcription, use of the LTR, RNA expression, and production of virus particles appear to be as efficient in U937 cells as in H9 cells. We have also compared viral growth characteristics in H9 and THP-1 cells. The latter is another human monocytic cell line, which also contains more surface CD4 than H9 cells (38). Again, HIV-1-WI3 grew poorly in THP-1 and its growth was blocked before accumulation of viral DNA (unpublished data), indicating that our observation was not restricted to one cell line. Also, no viral DNA accumulation over 96 h was evident when peripheral macrophages were infected with HIV-1-WI3 (unpublished data).

It was recently reported that some mutations in the *env* region of a T cell-tropic HIV strain could abolish the already inefficient viral growth seen in monocytes (U937), without altering the efficient growth in T cells (SUP-T1), and that CD4 levels in these cell lines were comparable (4). Together with these observations, our results suggest that viral entry is the limiting factor for viral growth in monocytes. This is somewhat surprising, because U937 cells have significantly more CD4 on the cell surface than H9 cells. One possible explanation for this is that factors other than CD4 are involved in viral entry. For example, CD4 may have to interact with other molecule(s), or the HIV *env* product may have to interact with a second cellular protein after binding to CD4. Such additional host component(s) may react differently with viral strains showing different tropisms. A further possibility is that the CD4 molecule of monocytes may differ from that of T cells, perhaps by posttranslational modification. These explanations are also supported by the observations that CD4-expressing human and murine cells do not support HIV growth even though the virus may bind to the cell (3, 28).

There are certain HIV isolates, such as HIV-1_{Ba-L}, that grow more rapidly in monocytes/macrophages than in T cells (14). Such viruses must be able to overcome the inefficient viral entry to monocytes seen with the T-cell tropic strains. Because the Env protein interacts with the CD4 receptor, studies on viral factors determining tropism have concentrated on this gene. For example, an HIV strain tropic for T cells could grow in monocytes after replacement of a nucleotide sequence that included the *env* region by that from a strain tropic for monocytes/macrophages (11). However, Env is not the only factor determining the specificity of viral entry, because many HIV isolates tropic for monocytes/macrophages do not grow well in T cells which have abundant CD4 (9, 14). We have recently found that the lack of growth in T cells of HIV_{Ba-L}, a strain tropic for primary macrophages, is likewise probably due to the inefficiency of processes occurring between viral entry and reverse transcription (unpublished data).

It has been shown in a number of independent experiments that certain viral strains do not grow well in cells with high levels of CD4 on the cell surface. These include inefficient or no growth of macrophage-tropic viral strains in T-lymphoid and monocytic cell lines containing high levels of CD4 (1, 14, 25), of T-cell-tropic strain in nonreplicating primary T cells (7, 17), and of unamplified viral strains (i.e., virus that has been not been passaged in cell lines in vitro after its initial isolation from patients) in CD4-positive lymphoid cell lines (9). Previously, one of the most likely explanations for such inefficient viral growth was thought to be at the level of gene expression (9, 12). However, we have found that in the cases described above, inefficient viral growth is determined during the early stages of the virus life cycle despite high levels of CD4 on the cell surface. These observations indicate that the process of viral entry is the result of complex interactions between host and viral factors in addition to CD4 and gp120 molecules. Analyzing these interactions is a key step to understanding the pathogenetic mechanism of HIV.

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