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Infection of Nonlymphoid Cells by Human Immunodeficiency Virus Type 1 or Type 2

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Human epithelial cells (L132) derived from embryonic lung and human lung fibroblasts (MRC5) were infected by human immunodeficiency virus type 1 (HIV-1) or type 2 (HIV-2). Surface CD4 protein was detected on these cells, and recombinant soluble CD4 (sCD4) blocked infection, indicating that HIV infection was mediated by the cell surface CD4 protein. In contrast, infection of human primary chondrocyte cells (C23), synovial cells (HSA), and foreskin fibroblasts (F13) was apparently independent of cell CD4-mediated mechanisms. Surface CD4 protein could not be detected on these cells, and sCD4 did not block the infection. F13 cells could be infected only by HIV-2, not by HIV-1, under our experimental conditions. In cells of mesenchymal origin, viral production could be detected only after cocultivation with the human T-lymphoid H9 cells but not by conventional viral assays, including reverse transcriptase and p24 antigen assays in cell culture supernatant and immunofluorescence of host cells. Our DNA transfection studies indicated that this lack of detectable viral production was not due to the inefficient use of the HIV long terminal repeat or the Tat protein in these cells. These mesenchymal and epithelial cells were susceptible to HIV infection but differed in mechanism of virus entry compared with hematopoietic cells such as T lymphocytes. These observations may provide insights into clinical syndromes such as lung dysfunction in HIV-infected newborns and connective tissue disorders in HIV-infected adults.

Certain human immunodeficiency virus (HIV)-infected individuals have clinical manifestations that cannot be explained solely by their depletion of $CD4^+$ T cells. Examples of these conditions include HIV-infected newborns with lung dysfunction due to an unexplained interstitial pneumonia (3, 5, 31) and adult patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex who have nonspecific arthritis or arthralgia (2, 12, 22, 29, 37). It has been proposed that HIV may be able to infect certain cellular components of the lung or joints, and such infection could contribute to these clinical disorders.

HIV is known to infect nonlymphoid cells, including macrophages (13, 20, 30), colorectal epithelial cells (1, 28), neuroglial cells (6, 7, 36), and cutaneous fibroblasts (34). The infection of T-lymphoid cells is dependent on the surface CD4 molecule, which functions as the viral receptor (9, 24). Both CD4-dependent and -independent mechanisms of nonlymphoid cell infection have been suggested (8, 19, 34).

To better understand the role of nonlymphoid cells in the pathogenesis of HIV disorders, we studied HIV infection of epithelial and mesenchymal cells, which are important components of lung and joints. We found that both epithelial and mesenchymal cells derived from the lung could be infected by HIV and utilized the surface CD4 molecule as a viral receptor. In addition, primary human synovial cells, chondrocytes, and foreskin fibroblasts were also susceptible to HIV infection. The infection of these cells appeared to be independent of the cellular CD4 molecule.

Our results suggested that these nonlymphoid cells have the potential to serve as reservoirs for HIV. Furthermore, direct infection of nonlymphoid tissues by HIV should also be considered in the pathogenesis of lung dysfunction and of connective tissue disorders. Our observations also revealed that certain mesenchymal cells could be protected from HIV infection by recombinant soluble CD4 (sCD4), an antiviral agent entering clinical trials, whereas others would not be protected. These studies demonstrate the complexity of the interaction between HIV and the host and emphasize the importance of evaluating potential antiviral agents in a variety of experimental cell systems.

MATERIALS AND METHODS

Cells. L132 cells (ATCC CCL5) were derived from normal human embryonic lung and grown in medium 199 (GIBCO Laboratories) supplemented with 10% fetal bovine serum (GIBCO) plus penicillin and streptomycin. MRC5 cells (ATCC CCL171) were derived from fetal pulmonary fibroblasts and grown in Dulbecco modified Eagle medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum plus penicillin and streptomycin. Primary cultures of foreskin fibroblasts (F13), chondrocytes (C23), and synovial cells (HSA) were obtained by serial passage of minced fresh tissue and carried in Dulbecco modified Eagle medium with 10% fetal bovine serum plus penicillin and streptomycin (14, 15). Cells were passaged more than 10 times before the described experiments.

All adherent cells were documented in our laboratory to be mycoplasma free (GEN-PROBE). To characterize the cell types, monoclonal antibodies to the protein vimentin or cytokeratin (Labsystems) were used in an indirect immunofluorescence assay (IFA). These proteins were checked by flow cytometry analysis (FACS; Becton Dickinson and Co.), using anti-Leu3a monoclonal antibody (Becton Dickinson), which recognizes the binding site of HIV to CD4 protein. In

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studies of infectibility, the stock cell cultures were removed with 0.5% trypsin-EDTA (GIBCO), washed, and plated at a cell density of $2 \times 10^4/cm^2$ once or twice a week.

Virus stocks. The H9 lymphoid cell line (a gift of R. Gallo, National Cancer Institute) was used to grow the HIV-1_{IIIB} isolate and the HIV-2_{ROD} isolate (a gift of J. Chermann, Institut Pasteur) by the shake method of Vujcic et al. (35). Viral stocks were filtered through a 0.45-μm-pore-size filter (Nalgene) to prevent contamination with H9 cells.

Virus was stored at −70°C until used for experiments. The titer of the viral stocks was determined by serial dilution on H9 cells. The 50% tissue culture infective dose ($TCID_{50}$) was defined as the amount of virus that resulted in half of the cultures demonstrating detectable viral infection, i.e., supernatant reverse transcriptase (RT) activity greater than 2 standard deviations above background, after 7 days in culture. Virus production was measured by supernatant RT activity, HIV core p24 antigen (Abbott Laboratories) production, and IFA (17).

HIV infection of cells. For infection of adherent cells with HIV-1_{IIIB} or HIV-2_{ROD}, 5×10^5 cells were plated onto 25-cm^2 flasks (Corning Glass Works) 24 h before infection. Cells were treated with DEAE-dextran (25 μg/ml; Sigma) to enhance viral absorption (10, 25) for 30 min at 37°C, washed with serum-free medium, and then incubated with 4 ml of media containing different amounts of virus, as indicated. After 24 h, cells were trypsinized, washed, and seeded onto new flasks. When cells reached confluence every 3 or 4 days, they were trypsinized and washed. On days 10 to 15 after infection, H9 cells were added to the cultures as target indicator cells for HIV infection. After 24 h, H9 cells were harvested and seeded onto 24-well plates at a cell density of 2×10^5/ml. Viral production was monitored by IFA, RT assay, or HIV p24 antigen assay for 2 weeks after addition of H9 cells.

Inhibition of HIV infection by recombinant sCD4 or anti-Leu3a antibody. To study blocking of infection by sCD4, cells were exposed to preparations of HIV-1_{IIIB} or HIV-2_{ROD} that were pretreated with different concentrations of sCD4 for 1 h at 4°C. In other experiments, cells were incubated with anti-Leu3a antibody (5 or 0.05 μg/ml) at 37°C for 1 h before incubation with virus. Recombinant sCD4 (a gift of T. Gregory, Genentech, Inc.) was cloned and expressed in mammalian cells as described previously (4, 32). The protein was >98% pure by silver stain after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and was diluted in sterile phosphate-buffered saline. In the sCD4 or Leu3a blocking experiments, the adherent cells were not treated with DEAE-dextran before infection.

Plasmid DNA, transfection, and CAT assays. pHIV-LTR-CAT was constructed by ligating the *Kpn*I-*Hin*dIII fragment of the HIV-1 long terminal repeat (LTR) into the *Pvu*II-to-*Hin*dIII site of pSV918. This construct was identical to pSVECAT (16) except that the pBR322 backbone was replaced by SP65 (27). pHIV-2-LTR-CAT, pSV-HIV-1-TAT, and pSV-HIV-2-TAT were described by Emerman et al. (11). Transfection of these plasmids was performed by the DEAE-dextran technique (33) as modified by Grosscheld and Baltimore (18) for H9 cells and by Lopata et al. (26) for adherent cells. Cell extracts were prepared for chloramphenicol acetyltransferase (CAT) assay 48 h after transfection. CAT activity was determined as described previously (11, 16).

Metabolic labeling and radioimmunoprecipitation. HIV-infected cells were cultured in 10-cm dishes (Nunc), incubated in 10 ml of selective medium lacking methionine and cysteine for 2 h at 37°C, and then labeled by addition of 400 μCi of [^{35}S]methionine and 400 μCi of [^{35}S]cysteine (Dupont, NEN Research Products) for 24 h at 37°C. After labeling, cells were washed with phosphate-buffered saline (pH 7.4) and lysed in NTE buffer (100 mM NaCl, 20 mM Tris hydrochloride, 10 mM EDTA) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors (10 μg of pepstatin A, 10 μg of leupeptin, 10 μg of aprotinin, and 10 μg of trypsin inhibitor per ml plus 10 μM benzamidine). Then 100 μl of lysate, cleared by microcentrifugation for 10 min at 4°C, was incubated with an appropriate antibody for 4 h at 4°C and absorbed onto protein A-Sepharose 6MB (Pharmacia) for 1 h at 4°C. After samples were washed and boiled in a buffer containing SDS and β-mercaptoethanol, they were analyzed by electrophoresis through a 12.5% SDS-polyacrylamide gel (23).

RESULTS

To ensure that cells used in these studies were indeed those of the respective cell types, the cells were initially characterized by surface immunofluorescence, using monoclonal antibodies to the protein cytokeratin or vimentin, both of which are cell markers of epithelial or mesenchymal cells. Cytokeratin was detected only on L132 cells, whereas vimentin was detected on foreskin fibroblasts (F13), chondrocytes (C23), pulmonary fibroblasts (MRC5), and synovial cells (HSA). L132 cells were thus shown to be epithelial in origin, and F13, MRC5, C23, and HSA cells were confirmed to be mesenchymal in origin. The presence of surface CD4 protein was also determined by IFA and FACS analysis, using anti-Leu3a, an anti-CD4 monoclonal antibody. Microscopically, CD4 protein was not detected by IFA on the cell surface of any of the cell lines. FACS analysis demonstrated that approximately 8% of L132 cells and 4% of MRC5 cells had detectable surface CD4 expression above background. No surface CD4 protein was detected on F13, C23, or HSA cells by FACS analysis.

Experiments of HIV infection of L132 cells and of inhibition of infection of these cells by sCD4 were performed. HIV-1 was able to infect L132 cells over a range of virus concentrations (25 to 800 $TCID_{50}$; Fig. 1A). About 20% of L132 cells expressed HIV protein by IFA 22 days after infection. In long-term experiments, this percentage of antigen-positive cells gradually increased to a maximum of >80% after 30 days (results not shown). L132 cells did not manifest cytopathic changes after HIV infection. Virus production in these cultures was readily detected by supernatant RT activity and specific radioimmunoprecipitation of HIV proteins (Fig. 1B). The origin of the extra virus-specific band in the p24 region is not known, but it was not observed in control H9 cell immunoprecipitations. sCD4 concentrations of 1 μg/ml blocked the infection of L132 cells by HIV-1, as did anti-Leu3a (Fig. 2A). sCD4 or anti-Leu3a also blocked the transfer of HIV infection from infected L132 cells to uninfected H9 cells (Fig. 2B). These results show that HIV-1 infection of L132 cells was dependent on the CD4 receptor molecule.

Similar studies of HIV infection and of inhibition of infection by sCD4 were performed with the cells of mesenchymal origin, MRC5, F13, C23, and HSA cells. Virus production in mesenchymal cells alone could not be detected by analyses of supernatant RT activity, p24 antigen production, and IFA. HIV infection of mesenchymal cells was detected only after cocultivation with T-lymphoid H9 cells. Human fetal lung fibroblasts (MRC5) could be infected by

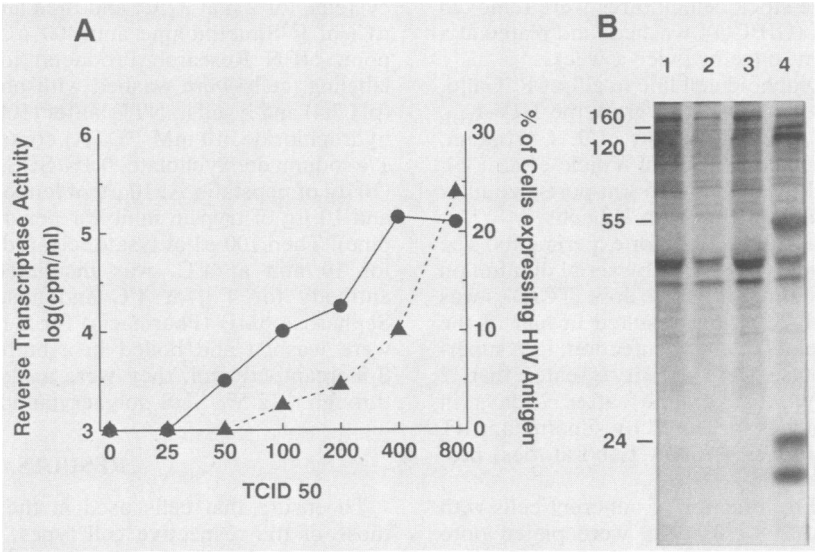

FIG. 1. HIV-1 infection of L132 cells. After exposure of cells to different titers of virus, virus production was detected by supernatant RT assay (●) and IFA (▲) 22 days after infection (A) and by radioimmunoprecipitation assay and SDS-PAGE analysis (B). L132 cells infected by HIV-1 were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to radioimmunoprecipitation and SDS-PAGE analysis, using an anti-HIV-seropositive serum (lane 4) or an HIV-seronegative serum (lane 2). As a control, uninfected L132 cells labeled with [^{35}S]methionine and [^{35}S]cysteine were analyzed with the anti-HIV serum (lane 3) and the negative serum (lane 1).

either HIV-1 or HIV-2 (Fig. 3B). Only HIV-2, not HIV-1, was able to infect foreskin F13 cells (Fig. 3A).

Primary chondrocytes (C23 cells) and primary synovial cells (HSA cells) were inoculated with HIV-1 or HIV-2 (Fig. 4). Again, virus production was detected only after cocultivation with H9 cells, even when cells were cultured for 40 days. Synovial cells were susceptible to HIV-2 infection but not HIV-1 infection under these conditions.

In the above-mentioned HIV infection, host cells were pretreated with DEAE-dextran, which has previously been used to enhance viral absorption (10, 25). To avoid a possible artifact that may result from its use, we also studied susceptibility of these cells to HIV infection without pretreatment with DEAE-dextran. The results were similar to those described above except that HIV-1 infection of C23 or HSA cells could not be detected, which suggested that the use of agents like DEAE-dextran may have enhanced infection of these cells by HIV-1.

Next, we tested the inhibitory effect of sCD4 on infection of these mesenchymal cells (Table 1). In these blocking experiments, to prevent any potential interfering effects of DEAE-dextran on the inhibitory properties of sCD4, the

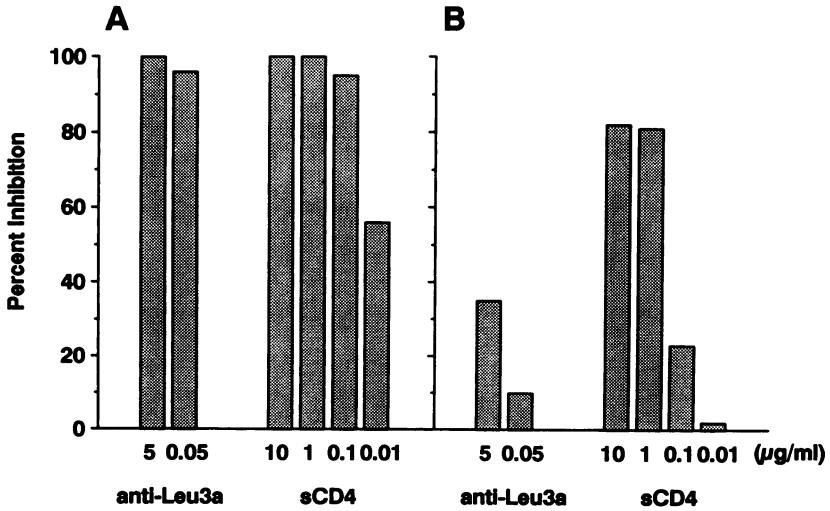

FIG. 2. (A) Inhibitory effect of sCD4 or anti-Leu3a on HIV infection of L132 cells. L132 cells were treated with anti-Leu3a (5 or 0.05 μg/ml) for 1 h at 37°C, or HIV (2,000 $TCID_{50}$) was treated with different concentrations of sCD4 for 1 h at 4°C before infection. Virus production was detected by RT assay 7 days after infection. (B) Inhibition by sCD4 or anti-Leu3a of the transfer of HIV infection from infected L132 cells to uninfected H9 cells. Uninfected H9 cells were added to infected L132 cells with several concentrations of sCD4 or anti-Leu3a. After 24 h, H9 cells were harvested and put on 24-well plates, and virus production was measured by RT assay. Percentage inhibition was measured by RT activity versus control RT activity.

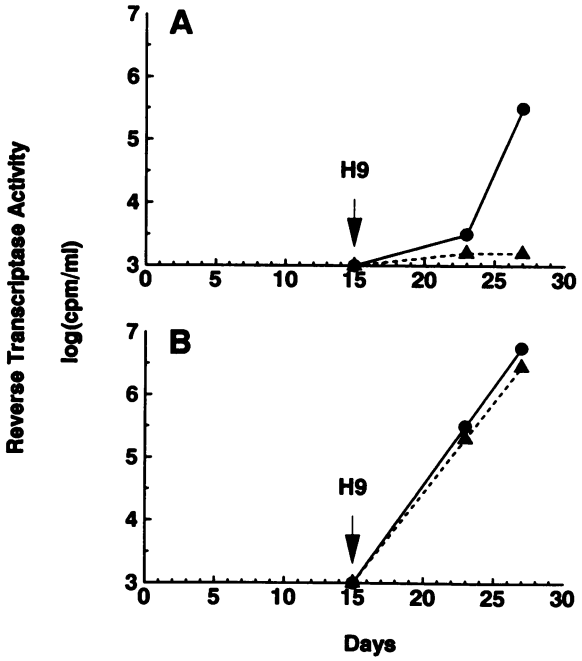

FIG. 3. Infection of F13 (foreskin fibroblasts) (A) or MRC5 (fetal lung fibroblasts) cells (B) by HIV-1_{IIIB} (▲) or HIV-2_{ROD} (●). Cells were trypsinized, washed, and placed in new flasks 24 h after exposure to virus (2,000 $TCID_{50}$). When cells were confluent, they were subcultured every 3 or 4 days. H9 cells were added to these cells 15 days after inoculation. After 24 h, H9 cells were harvested and put into 24-well plates. Virus production was measured by RT assay and IFA (not shown).

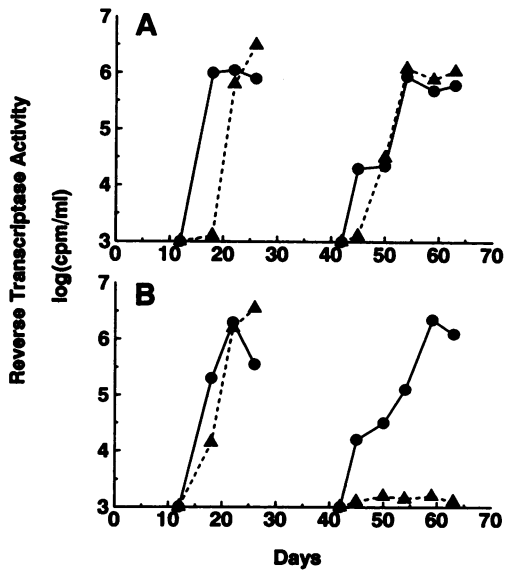

FIG. 4. Infection of C23 (chondrocytes) (A) or HSA (synovial cells) (B) cells by HIV-1_{IIIB} (▲) or HIV-2_{ROD} (●). H9 cells were added to cells 12 and 42 days after inoculation with virus. Virus production was measured by RT assay and IFA (not shown).

cells were not pretreated with DEAE-dextran. Recombinant CD4 blocked the infection of MRC5 cells by HIV-1 or HIV-2. The concentrations of sCD4 required to block infection by HIV-2 were 5- to 10-fold higher than those required to block HIV-1 infection, as previously observed for T cells in other systems (4, 8). HIV infection of MRC5 cells thus appeared to be dependent on the CD4 receptor.

On the other hand, HIV-2 infection of foreskin fibroblasts, chondrocytes, or synovial cells was not inhibited by sCD4, even at concentrations of 40 μg/ml. Inhibition experiments with HIV-1 were not performed because this strain did not cause infection under these conditions. Because these mesenchymal cells did not express membrane CD4 and were not protected from infection by sCD4, it appears that the observed HIV infection was independent of the CD4-mediated pathway.

As shown above, the cells of mesenchymal origin could be infected by HIV. Virus production in these cells was undetectable by conventional assays and could be detected only after cocultivation with the T-lymphoid H9 cells. To study whether lack of detectable virus production in mesenchymal cells was determined by the HIV LTR, we analyzed expression of the CAT gene linked to the HIV-1 or HIV-2 LTR in F13 and C23 cells (Fig. 5). To estimate the relative strength of the HIV LTR and its transactivation by Tat, the same plasmids were transfected into H9 cells and THP-1 cells, and CAT activities were measured under the same conditions (i.e., the same amount of plasmids and the same reaction conditions). Under our conditions, the level of CAT activity driven by the HIV LTR alone was close to the background level, less than 0.1% conversion of chloramphenicol to acetylated forms. However, cotransfection of the HIV LTR CAT constructs and respective Tat expression plasmids resulted in a significant increase in the level of CAT activity. It is difficult to directly compare the level of CAT activity between different cell types because of a difference in the pattern of cell growth and complexity in measurement of transfection efficiency, but CAT activities in F13 and C23 cells were always readily detectable. These results indicated that the HIV LTR was active in these mesenchymal cells and was efficiently transactivated by the respective Tat protein. We reproducibly observed that expression of the CAT gene driven by the HIV-2 LTR was higher than that driven by the HIV-1 LTR.

DISCUSSION

Considerable interest exists in defining the full spectrum of target cells of HIV infection and in the comparative biology

TABLE 1. Inhibitory effect of sCD4 on infection of mesenchymal cells by HIV-1 or HIV-2[a]

Cell line	Virus	Inhibition at sCD4 concn (μg/ml) of[b]:			
		40	10	1	0.1
MRC5	HIV-1	+ (100)	+ (100)	+ (75)	−
	HIV-2	+ (91)	−	−	−
F13	HIV-2	−	−	−	ND
C23	HIV-2	−	−	−	ND
HSA	HIV-2	−	−	−	ND

[a] Virus (2,000 $TCID_{50}$) was preincubated with 4 ml of the indicated concentrations of sCD4 for 1 h at 4°C and then exposed to cells for 24 h at 37°C. Cells were not pretreated with DEAE-dextran. H9 cells were added to the adherent cells 10 to 15 days after exposure to virus. Viral production of the added indicator cells was followed by IFA, RT assay, and HIV p24 antigen assay. Positive control infections showed transfer of infection to H9 cells within 10 days in all cases.

[b] +, Inhibition of infection by sCD4; −, no inhibition. Percent inhibition (given in parentheses) was calculated by comparing the RT activities of test cultures and positive control cultures approximately 10 days after addition of H9 cells. Each experiment was performed two or three times except for the HSA infection, which was performed once. ND, Not determined.

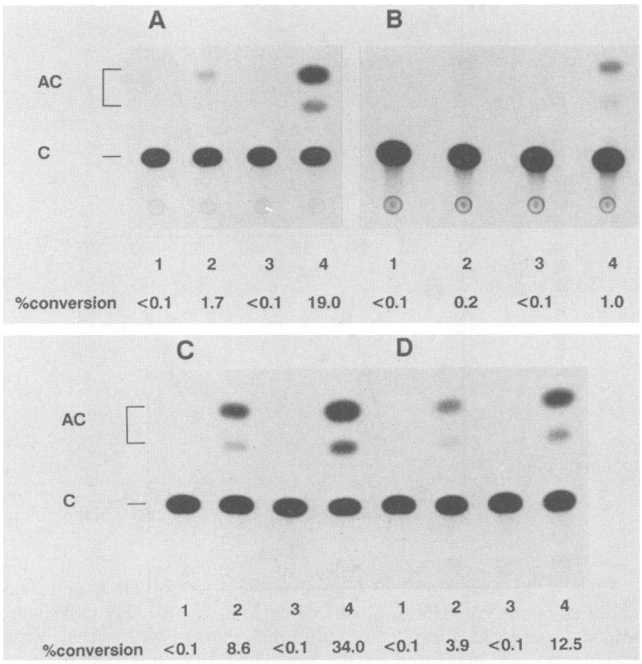

FIG. 5. CAT analysis of F13 (foreskin fibroblasts) (A), C23 (primary chondrocytes) (B), T-lymphoid H9 (C), and monocytic THP-1 (D) cells transfected with pHIV-1-LTR-CAT alone (lanes 1), pHIV-1-LTR-CAT and pHIV-1-TAT expression plasmids (lanes 2), pHIV-2-LTR-CAT alone (lanes 3), or pHIV-2-LTR-CAT and pHIV-2-TAT (lanes 4). Cells were transfected by the DEAE-dextran procedure, and CAT analysis was performed as described in the text. The values represent absolute percent conversion of [^{14}C]chloramphenicol to its acetylated forms. C, Unreacted substrate ([^{14}C]chloramphenicol); AC, acetylated forms. Background level was less than 0.1% conversion of chloramphenicol to acetylated forms.

of HIV entry and replication in different cell types. We studied infection of epithelial cells or mesenchymal cells by HIV-1 or HIV-2. In our experiments, L132 epithelial cells and MRC5 fibroblasts derived from human fetal lung were susceptible to infection by HIV-1 or HIV-2. The CD4 molecule, a receptor for HIV, was detected on these pulmonary cells, and recombinant CD4 or anti-Leu3a blocked both primary infection and transfer of virus to lymphoid cells. This result supports a CD4-dependent mechanism of infection of L132 or MRC5 cells.

Several mesenchymal cells of different origin, including foreskin fibroblasts, chondrocytes, and synovial cells, were also studied with respect to susceptibility to infection by HIV. These cells had no detectable surface CD4 protein but could be infected by HIV. Recombinant CD4 failed to block the infection of CD4-negative mesenchymal cells. Infection of these mesenchymal cells did not appear to depend on the CD4 molecule. This observation strongly supports the likelihood of the existence of another mechanism for virus infection independent of the surface CD4 molecule. Recently, it was reported that CD4-independent infection by HIV-1 may occur in osteosarcoma cell lines and other cell types (8, 19, 21, 34).

In our experiments, HIV-2 more readily infected these mesenchymal cells than did HIV-1. All of the cell types were infected by HIV-2 in the presence or absence of DEAE-dextran, whereas DEAE-dextran was required for HIV-1 infection of C23 or HSA cells. Although the function of this agent is not clear, it is likely to be involved in one of the steps of viral entry, such as binding, fusion, or uncoating. If this is the case, HIV-2 virions may include some component that permits more efficient entry into non-CD4 cells.

Infected mesenchymal cells did not produce enough cell-free virus to be measured directly by the standard RT assay or even the more sensitive p24 antigen capture assay. After long-term culture and a number of trypsinizations, virus was detected only after the infection was passed to the indicator T-cell line, H9. We do not know whether this result was due to restricted replication in many cells or normal expression in very rare infected cells. The results of our DNA transfection studies suggested that the HIV LTR was reasonably active relative to its activity in H9 cells and other monocytic cells (THP-1 cells) and that the Tat protein can transactivate the HIV LTR as efficiently as in more susceptible host cells. Therefore, it is not likely that these two factors, the HIV LTR and Tat, are major factors in determining the level of virus production in mesenchymal cells. Further studies will be required to identify the cellular and viral factors that are responsible for this result.

In this report, we demonstrated that primary synovial cells, like C23 cells, could be infected by HIV in vitro. Consistent with this observation is the report that HIV has been isolated from the synovial fluid of patients with severe joint pain (37). The infection of synovial cells or chondrocytes by HIV may contribute to the rheumatological syndromes in HIV-infected patients. Infection of these cells did not appear to require surface CD4 and was not blocked by recombinant CD4, suggesting that recombinant CD4 may not be useful for the treatment of this particular HIV-related manifestation. Investigation of primary mesenchymal cells derived from patients with HIV infection should be performed to better define the biology and clinical importance of retroviral infection of these cell types.

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