A Mechanism of Restricted Human Immunodeficiency Virus Type 1 Expression in Human Glial Cells

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We characterized in detail the life cycle of human immunodeficiency virus type 1 (HIV-1) in human glioma H4/CD4 cells which stably express transfected CD4 DNA (B. Volsky, K. Sakai, M. Reddy, and D. J. Volsky, *Virology* 186:303–308, 1992). Infection of cloned H4/CD4 cells with the N1T strain of cell-free HIV-1 (HIV-1/N1T) was rapid and highly productive as measured by the initial expression of viral DNA, RNA, and protein, but all viral products declined to low levels by 14 days after infection. Chronically infected, virus-producing H4/CD4 cells could be obtained by cell cloning, indicating that HIV-1 DNA can integrate and remain expressed in these cells. The HIV-1 produced in H4/CD4 cells was noninfectious to glial cells, but it could be transmitted with low efficiency to CEM cells. Examination of viral protein composition by immunoprecipitation with AIDS serum or anti-gp120 antibody revealed that HIV-1/N1T-infected H4/CD4 cells produced all major viral proteins including gp160, but not gp120. Deglycosylation experiments with three different glycosidases determined that the absence of gp120 was not due to aberrant glycosylation of gp160, indicating a defect in gp160 proteolytic processing. Similar results were obtained in acutely and chronically infected H4/CD4 cells. To determine the generality of this HIV-1 replication phenotype in H4/CD4 cells, nine different viral clones were tested for replication in H4/CD4 cells by transfection. Eight were transiently productive like N1T, but one clone, NL4-3, established a long-lived productive infection in H4/CD4 cells, produced infectious progeny virus, and produced both gp160 and gp120. We conclude that for most HIV-1 strains tested, HIV-1 infection of H4/CD4 is restricted to a single cycle because of the defective processing of gp160, resulting in the absence of gp120 on progeny virus.

The majority of AIDS patients develop primary central nervous system (CNS) diseases attributed directly to human immunodeficiency virus type 1 (HIV-1) infection (29, 51, 62, 69, 81). The most common of these, known as HIV-1-associated encephalopathy in children and AIDS dementia complex in adults, is characterized by progressive neuronal loss and brain atrophy (9, 29, 61, 79, 82). HIV-1 invades the brain early in HIV-1 infection (17, 46, 81), and viral levels in the central nervous system often correlate with the severity of CNS diseases (6, 8, 95), suggesting a causative role of the virus. The cellular and virological mechanisms responsible for the neurological damage in AIDS have not been resolved. Analyses of brain autopsy materials from AIDS patients indicate that HIV-1 persists at high levels in macrophages and microglial cells (80, 86, 98). Both of these cell types are susceptible to highly productive HIV-1 infection *in vitro* (33, 34, 94). It has been proposed that these HIV-1-infected cells contribute to neuronal damage indirectly by secreting cellular and viral neurotoxic products (19, 24, 35, 36, 71). On the other hand, increasing evidence suggests that HIV-1 also can infect cells of the neuroectodermal origin, including astrocytes and neurons, as well as other brain cells, raising the possibility that HIV-1 infection contributes directly to the nervous system dysfunction in AIDS. Transformed glial and neuronal cells (11, 13, 21, 49, 56, 78), primary astroglial cells (11, 60, 89, 97), brain-derived

microvascular endothelial cells (57, 68), untransformed cortical cells with neuronal precursor cell characteristics (55, 90), and, most recently, primary neuroblasts from fetal olfactory epithelium (28) can be infected with HIV-1 *in culture*, although such infections are generally of low productivity and noncytopathic. In early studies, HIV-1 genomes and proteins rarely were detected in astrocytes and neurons in postmortem brain tissue (86, 98), but recent studies with sensitive detection methods indicate that HIV-1 infection in such cells *in vivo* can be quite prevalent, and it often is correlated with the severity of the CNS dysfunction (64, 70, 73, 87). For example, analysis of brain tissue from five adult and two pediatric AIDS patients by a combined *in situ* PCR (4) for the detection of the HIV-1 genome and by immunocytochemistry for the detection of cell-type-specific markers revealed HIV-1 DNA in up to 3% of astrocytes and 17% of neurons in all of the samples tested (64). Patients with severe dementia had the highest frequency of HIV-1-infected neural cells (64). A study of brain tissues from 12 pediatric AIDS patients with modified *in situ* hybridization and immunocytochemistry demonstrated the HIV-1 genome and Nef protein in 1% of astrocytes in subcortical white matter from four patients with advanced leucoencephalitis (87), while a similar study of postmortem tissue from six children who died with AIDS encephalopathy revealed the presence of Nef mRNA and protein in up to 20% of astrocytes in some sections (73). Rev and Nef proteins were also found in astrocytes in postmortem brain tissues from adult AIDS patients (70), while the most recent study confirmed low-level infection of astrocytes but not neurons in adult AIDS patients' brains (86a). Consistent with studies *in vitro* (21, 60, 89, 97), most of the tissue samples in which virus expression was examined showed low-productivity or latent HIV-1 infection (64, 73).

We have been interested in the mechanism of restricted

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HIV-1 replication in human glial cells in vitro as a model for direct interaction of HIV-1 with nervous system-derived cells. In general, the pattern of HIV-1 infection of these cells in vitro differs radically from that in T lymphocytes (93). Cultured glial cells and primary astrocytes generally do not express surface CD4 (10), and HIV-1 infects these cells by a CD4-independent route (15, 16, 38, 56). Galactosylceramide serves as an HIV-1 receptor on some glial cells (5, 37). HIV-1 has been shown to fuse with (56) and enter (39, 56) CD4-negative glial cells in vitro, but viral expression in these cells is about 3 orders of magnitude less efficient than that in T cells, and no cytopathic effects are observed (39, 48, 56, 91). Transfection of viral DNA into glial cells permitted a more robust HIV-1 expression than in cells exposed to cell-free virus (21, 77, 85, 89), indicating that the CD4-independent mode of virus entry is one limiting factor in HIV-1 infection in these cells. However, both stable and transient HIV-1 DNA transfectants appeared to extinguish virus expression over time (21, 78, 88, 89), suggesting that additional negative regulatory mechanisms after HIV-1 entry contribute to the restricted infection in these cells. The dormant HIV-1 genome in such long-term astrocytic cultures can be induced by agents known to activate long terminal repeat-mediated gene expression, such as sodium butyrate (77) or tumor necrosis factor alpha and interleukin 1β (88, 89), indicating that these cells restrict HIV-1 expression at the level of long terminal repeat-mediated transcription. Another level of cellular regulation of HIV-1 expression has been described in the chronically infected TH4-7-5 glial cell line derived from HIV-1-infected 85HG-66 glioma cells (7). These cells exhibit a cell-determined block of HIV-1 Rev/RRE function (45) which contributes to the observed low levels of viral structural proteins and elevated levels of Nef (63).

The inefficient HIV-1 infection of glial cells in vitro and in vivo hinders studies of the regulation of the HIV-1 life cycle in these cells. In an attempt to circumvent this problem, we transfected human glioma H4 cells (HTB148) with human CD4 cDNA (53) and selected cells which stably express high levels of human CD4 (92). Coculture of stable transfectants, termed H4/CD4, with HIV-1-positive T cells permitted efficient virus replication. Surprisingly, HIV-1 expression in H4/CD4 cells was transient and declined to low levels typical of CD4-negative glial cells within 2 to 3 weeks after infection (92). In this report, we present detailed characterization of the restricted HIV-1 life cycle in these CD4-positive glial cells. Using molecular clones of HIV-1 and subclones of H4/CD4 cells which permit efficient infection with cell-free HIV-1, we show that these cells support efficient but transient synthesis of HIV-1 DNA and RNA and permit viral DNA integration. Examination of viral protein production revealed a defect in the cleavage of the envelope precursor glycoprotein gp160. Of nine virus strains examined by transfection into H4/CD4 cells, eight showed the efficient, but transient replication phenotype. We conclude that infection of glial cells can be restricted by the production of progeny virus carrying only immature forms of the viral envelope glycoprotein.

MATERIALS AND METHODS

Cells and viruses. The establishment of the glial cell line H4/CD4 has been described previously (92). Briefly, human neuroglioma HTB148 cells were transfected with the vector pKS286, which expresses human CD4 under the control of the HIV-1/N1T-E long terminal repeat (36) and carries the bacterial neomycin resistance gene (Fig. 1). Cells were selected for resistance to G-418 and cloned by limiting dilution, and clones expressing high levels of surface CD4 and susceptible to efficient infection with cell-free HIV-1 were selected for further study. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 500 μ g of G-418 per ml. CR10 (10) and CEM cells were maintained in RPMI-1640 with 10% fetal calf serum. HIV-1 N1T-A

FIG. 1. Schematic map of the eucaryotic CD4 expression plasmid pKS286. SV40, simian virus 40; CMV, cytomegalovirus.

and NL4-3 were propagated in chronically infected T cells and harvested, and their titers for biological activity were determined as previously described (10, 36). The following infectious molecular clones of HIV-1 were used in this work (the provider is listed in parentheses and the reference and the tropism of each clone are listed in Table 2): YU-2 (B. Hahn), 89.6 and Z6 (A. Srinivasan), NLXADA-GP and -PG (L. Ratner), SF-2 (J. Levy), HXBc2 (J. Sodroski), and NL4-3 (M. Martin). N1T-A was cloned in this laboratory (74). HIV-1 clone Menv2 (obtained from R. Sadaie) has a stop codon in the HIV-1 envelope protein (72), and it was used as a control for nontransmissible HIV-1 in transfection experiments. Viral DNA was amplified in bacterial plasmids and purified for transfection by standard methods (3).

HIV-1 infection and transfection. H4/CD4 cells were harvested and resuspended with cell-free HIV-1 at 1 to 2 pg of p24 per cell, a multiplicity of infection (MOI) of 1 to 2 for T cells. After 2 h at 37°C, cells were washed and cultured under standard conditions. At the designated times after infection, cells were harvested for isolation of DNA and RNA, for p24 assay, or for metabolic labeling. Transfection of proviral DNA was conducted with standard calcium phosphate precipitation and 10 μ g of DNA per 2×10^6 cells (21, 32).

Hirt extraction and analysis of extrachromosomal HIV-1 DNA. At the indicated time points, extrachromosomal DNA was extracted by a modified Hirt procedure (41). After lysis and extraction, the total soluble DNA recovered from 2×10^6 to 5×10^6 cells was loaded onto a 0.9% agarose gel for electrophoresis. The same cell number was assayed in all samples at a particular time point. The separated DNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.) and analyzed for HIV-1-specific sequences by hybridization with a 32 P-labelled *Sac*I fragment derived from the HIV-1/N1G clone (74). Autoradiography was performed over 1 to 5 days with Kodak X-Omat film and intensifying screens.

Southern and Northern (RNA) blot analysis. Total cellular DNA or RNA was isolated from cells by the standard guanidinium isothiocyanate method (3) at the times indicated. Thirty micrograms of DNA was left undigested or was digested with *Sst*I and was analyzed by electrophoresis in a 1% agarose gel. Ten micrograms of RNA from each system was analyzed in a 1.2% agarose gel containing formaldehyde. Transfer, hybridization, and autoradiography were conducted as described above.

Radioimmunoprecipitation and deglycosylation. Approximately 12 h before each time point, HIV-1-infected and uninfected cells were washed twice with phosphate-buffered saline (PBS) and labeled overnight with 50 μ Ci of [35 S]methionine per ml (1,000 μ Ci/mmol; NEN, Boston, Mass.). At the end of the labeling period, cells were washed with PBS containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. Cell pellets (10^7 cells) were incubated in 1 ml

of immunoprecipitation buffer for 30 min at 4°C and centrifuged at $3,000 \times g$ for 20 min to remove nuclei. Immunoprecipitation buffer contained 0.02 M Tris-HCl (pH 3.0), 0.12 M NaCl, 0.2 mM PMSF, 5 µg of aprotinin per ml, 0.2 mM EGTA, 0.2 mM sodium fluoride, 0.2% sodium deoxycholate, and 0.5% Nonidet P-40 (NP-40). Cell lysates were preabsorbed with protein G-Sepharose beads (Pharmacia, Inc., Piscataway, N.J.) for 2 h at 4°C. Lysates were then incubated at 4°C for 2 h with 100 µl of protein G-Sepharose and 5 µl of either pooled serum from AIDS patients, sheep anti-human T-cell leukemia virus 3B gp120 antiserum (catalog number 288; AIDS Research and Reference Reagent Program) (see Fig. 9A), or goat anti-gp160 and -gp120 antiserum (catalog number 191, AIDS Research and Reference Reagent Program) (Fig. 9B). Conjugated beads were washed twice with a buffer containing 20 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 0.2% NP-40 and once with 50 mM Tris-HCl (pH 6.8). Washed beads were suspended in 100 mM sodium phosphate (pH 7.0)–0.1% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol and boiled for 3 min. Electrophoresis was performed in 8% or 10% polyacrylamide gels with a 4.5% stacking gel. Gels were fixed, dried, and autoradiographed with Kodak X-Omat film. Deglycosylation analyses were performed on immunoprecipitated proteins by two procedures. In the first experiment with glycopeptidase F (PNGase F) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), eluted proteins were adjusted to 1% NP-40–50 mM EDTA–0.5-µg/ml pepstatin–0.2-µg/ml leupeptin–1 mM PMSF. Samples were incubated at 37°C for 3 h in the presence or absence of 1 U of PNGase F. Samples were then resuspended in 2× polyacrylamide gel electrophoresis (PAGE) buffer (2.5% SDS, 80 mM Tris-HCl [pH 6.8], 5% β-mercaptoethanol, 0.008% bromophenol blue), incubated for 30 min at 37°C and 5 min at 100°C, and electrophoresed as described above. For comparison of three glycosidases, we used a procedure described by Jones et al. (42). Immunoprecipitates were boiled for 3 min in the presence of 0.15% SDS and β-mercaptoethanol and resuspended in a 35 mM phosphate buffer (pH 6.0) containing 2.5 mM CaCl₂, 1 mM PMSF, and 2% NP-40, the latter added to quench SDS. The samples were centrifuged, and supernatants were divided into aliquots and incubated at 37°C for 16 h with no further additions or after addition of PNGase F, neuraminidase (Sigma, St. Louis, Mo.), or endoglycosidase H (Endo H₁; New England Biolabs, Beverly, Mass.) at final doses, respectively, of 0.5, 0.1, and 20 U. Reactions were stopped by addition of concentrated reduced loading buffer, and samples were boiled for 3 min and electrophoresed as described above.

Sequencing of N1T-A envelope. Overlapping fragments from the N1T-A envelope gene were cloned into M13mp18 and M13mp19 vectors and sequenced in both directions by the dideoxyribonucleotide chain termination method (76). Reactions were catalyzed with modified T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions.

Other analytical assays. The levels of HIV-1 p24 core antigen were determined in culture supernatants or cell lysates with the Coulter HIV Ag kit (Hialeah, Fla.) according to the manufacturer's instructions. An indirect immunofluorescence (IF) assay for HIV-1 antigens was conducted with pooled AIDS sera as previously described (10). The IF assay for glial fibrillary acidic protein (GFAP) was conducted as previously described (21) with rabbit anti-human GFAP (Dako, Santa Barbara, Calif.).

RESULTS

Infection of CD4-expressing H4 glial cell clones with cell-free HIV-1. We have shown previously that H4/CD4 glial cells replicate HIV-1 efficiently after coculture with chronically infected T-cell lines, but not when exposed to cell-free virus (92). To facilitate the analysis of the HIV-1 life cycle in these cells, we cloned H4/CD4 by limiting dilution. Several subclones were identified which permitted efficient infection by both cell-free HIV-1 and cocultivation. Like the parental H4/CD4 cells (92), these subclones displayed GFAP and high levels of surface CD4 receptors; between 80 and 90% of the cells were CD4 and GFAP positive as determined by flow cytometry and by IF staining, respectively (not shown). A typical profile of HIV-1 infection with one of these clones, H4/CD4c22, is shown in Fig. 2. T-cell leukemia CEM cells served as a positive control. We evaluated the kinetics of expression of HIV-1 p24 core antigens by enzyme-linked immunosorbent assay (ELISA), the proportion of cells expressing HIV-1 antigens as determined by IF, and cell viability. The results show that within 3 days of infection with the same dose of cell-free HIV-1, the CD4-positive glial cells and T cells reached similar levels of intracellular p24, and in both cell populations, about 25% of the cells were IF positive. Although HIV-1 infection in CEM cells continued to expand, the proportion of IF-positive glial cells declined from its peak on day 3, and the expression of p24

FIG. 2. Infection of glial H4/CD4 and T-lymphoid CEM cells with HIV-1: cell viability and kinetics of viral antigen expression. Cells were infected with HIV-1/N1T at an MOI of 1 and analyzed at the indicated times as described in Materials and Methods.

decreased to barely detectable levels 14 days after infection. The viability of HIV-1-infected glial cells remained high throughout the period of follow-up (Fig. 2C), indicating that the observed decline in HIV-1 expression is not due to cell killing by the virus. Similar results were obtained in 10 experiments with different virus preparations. We conclude that H4/CD4 glial cells are susceptible to efficient infection with cell-free HIV-1, but that unlike T cells, the high level of HIV-1 expression is transient.

Kinetics of HIV-1 DNA accumulation in H4/CD4 cells. Transient viral expression may arise from instability of the viral genome in H4/CD4 cells or from inefficient spread of progeny virus throughout the target cell population. To investigate the status of viral DNA in HIV-1-infected H4/CD4 cells, we first analyzed unintegrated viral DNA over the course of infection by Hirt extraction and Southern blot hybridization (Fig. 3A). The peak of viral DNA synthesis was reached 12 h after infection in H4/CD4 cells, at which time CEM cells contained comparable amounts of HIV-1 DNA. Unintegrated HIV-1 DNA declined in both cell types 24 h after infection, but reached its peak 2 days later in CEM cells, similar to previous studies (44). In contrast, 3 days after infection, unintegrated DNA was undetectable in H4/CD4 cells, and they carried a very small amount of high-molecular-weight DNA which presumably is integrated.

To investigate the accumulation of HIV-1 DNA more thoroughly, total cellular DNA was isolated from infected H4/CD4 and CEM cells and analyzed by Southern blot hybridization (Fig. 3B). Consistent with the results of the previous experiment, 3 days after infection, CEM cells contained large amounts of high-molecular-weight HIV-1 DNA which generated the expected 8.9-kb fragment upon digestion with *Sst*I (Fig. 3B). Viral DNA was easily detected in H4/CD4 cells only 16 h after infection, and no viral DNA was detectable by this method 7 days after infection, consistent with the decline in viral expression in these cells (Fig. 2). These results indicate that H4/CD4 cells permit efficient HIV-1 entry, resulting in initially high levels of reverse-transcribed viral DNA, similar to that observed during the first cycle of infection of CEM cells.

Kinetics of viral RNA accumulation in H4/CD4 cells. To determine the transcriptional activity of the viral DNA present in H4/CD4 cells, we extracted RNA from infected cells at various times after HIV-1 infection and performed Northern blot analysis of viral RNA (Fig. 4). HIV-1 RNA expression was more rapid in infected H4/CD4 cells than in CEM cells; all

FIG. 3. Detection of HIV-1 DNA present in extrachromosomal and high-molecular-weight DNA fractions at various times after infection of H4/CD4 and CEM cells by HIV-1. (A) H4/CD4 (H) and CEM (C) were infected with HIV-1/N1T at an MOI of 1, and then extrachromosomal DNA was isolated at the time points indicated and subjected to Southern blot hybridization as described in Materials and Methods. (B) Cells were infected as described above, and high-molecular-weight DNA was extracted and analyzed after digestion with *Sst*I or without digestion (un) as described in Materials and Methods. M, DNA size markers; HMW, high-molecular-weight DNA; Lin. and Circ., linear and circular DNA forms, respectively.

three major size classes of HIV-1 RNA were present in infected glial cells but not in CEM cells 16 h after infection. High levels of viral RNA were detected in both cell populations 3 days after infection, but HIV-1 RNA levels decreased by 7 days in infected H4/CD4 cells, consistent with very low levels of

FIG. 4. Analysis of viral RNA expression in HIV-1-infected H4/CD4 and CEM cells by Northern blot hybridization. Cells from the experiment described in the legend to Fig. 3B were sampled at the same time points, and total cellular RNA was extracted and analyzed for the presence of HIV-1 transcripts by Northern blot hybridization as described in Materials and Methods.

FIG. 5. Comparison of HIV-1 DNA and RNA levels in chronically infected glial and T-lymphoid cells. High-molecular-weight DNA and total cellular RNA were isolated from chronically infected H4/CD4 clone H4/N1T-c12 and T-lymphoid CR10/N1T cells (10) at the time when more than 80% of both cell lines expressed HIV-1 antigens as determined by IF. *Sst*I-digested and undigested (un) DNA (A) and total cellular RNA (B) were probed for HIV-1 genomic sequences by Southern and Northern blot hybridization, respectively, as described in Materials and Methods.

HIV-1 DNA in these cells at that time. The proportions of the three major size classes of HIV-1 RNA were similar in CEM and H4/CD4 cells at days 3 and 7 after infection, indicating that transcription and splicing of viral RNA in H4/CD4 proceeded normally. Together, the findings of viral DNA, RNA, and protein accumulation (the latter as measured by IF and p24 production) all indicate that H4/CD4 glial cells, like CEM T cells, permit highly productive single-cycle infection by HIV-1; however, H4/CD4 infection is aborted after initial efficient viral expression, and infection in CEM cells continues to increase.

Chronic infection of H4/CD4 cells by HIV-1. To determine whether the cloned glial cell line used in these studies can support chronic HIV-1 infection, infected H4/CD4 cells were cloned by limiting dilution at the peak of HIV-1 expression and clones were evaluated for progression of HIV-1 infection by indirect IF staining for HIV-1 antigens. In two independent cloning experiments, about 5% of the clones stably expressed HIV-1 antigens. One of the IF-positive clones, H4/N1T-c12, was compared with a T-cell line, CR10/N1T, which is chronically infected by the same virus strain and produces high levels of viral proteins. The chronically infected glial cells were similar to the T-cell line in terms of viral DNA burden, as evaluated by Southern blot analysis of total cellular DNA or DNA after *Sst*I digestion (Fig. 5A). As determined by Northern blot analysis, H4/N1T-c12 cells also expressed the major viral transcripts, albeit at lower levels than CR10/N1T cells (Fig. 5B). These results indicate that abortive HIV-1 infection in H4/CD4 cells is not due to an intrinsic block to the HIV-1 life cycle prior to stable viral DNA integration or transcription in these cells.

Infectivity of the progeny viruses from H4/CD4. We have previously shown that continued viral DNA and protein synthesis by infected T cells in culture arises from multiple rounds of virus replication (67). The interruption of HIV-1 expression

TABLE 1. Infectivity of progeny virus produced in H4/CD4 glial cells^a

Virus source (mode of infection)	Target cells	HIV-1 expression after cell-free infection or cocultivation on day:					
		2		4		7	
		IF (%)	p24 (pg)	IF (%)	p24 (pg)	IF (%)	p24 (pg)
Expt 1 (cell-free virions)							
H4/CD4×N1T	H4/CD4	NT ^b	NT	<0.1	418	<0.1	15
H4/CD4×N1T	CEM	NT	NT	<0.1	430	0.5	3 × 10 ⁴
CEM×N1T	H4/CD4	NT	NT	21	1.5 × 10 ⁶	NT	NT
Expt 2 (cocultivation)							
H4/N1T-cl2	H4/CD4	18	850	5.0	1,000	<1	680
H4/N1T-cl2(ddC)	H4/CD4	15	1,000	4.0	1,300	<1	580
H4/N1T-cl2	CEM	<1	1,200	4.0	2,500	19	8.5 × 10 ⁴
H4/N1T-cl2(ddC)	CEM	<1	770	<1	460	<1	580
H4/N1T-cl2	H4/CD4	16	8,300	21	1.2 × 10 ⁴	21	2.3 × 10 ⁴
H4/N1T-cl2(ddc)	H4/CD4	11	8,800	<1	2,400	<1	1,000

^a In experiment 1, H4/CD4 or CEM cells were infected with concentrated cell-free N1T virus at the MOI as described in Materials and Methods. Three days after infection (when 24 and 50% of virus-infected H4/CD4 and CEM cells, respectively, expressed HIV-1 antigens as detected by IF), culture supernatants were collected, and virus in the supernatants was concentrated 50-fold and used for infection of H4/CD4 or CEM cells at an MOI of 0.1 as described in Materials and Methods. In experiment 2, chronically HIV-1 (strain N1T)-infected CR10/N1T and H4/N1T-cl2 cells (which were 85% and 89% IF-positive, respectively, at the time of the experiment) were growth arrested by treatment with 5 µg of mitomycin per ml and cocultivated at a 1:5 virus donor/acceptor ratio with the designated target cells. Virus expression was tested at the designated time points by IF and p24 ELISA. p24 data are expressed in picograms per 10⁶ cells in experiment 1 and picograms per milliliter of culture supernatant in experiment 2.

^b NT, not tested.

in H4/CD4 cells suggests that progeny virus produced during the first cycle of infection may be incapable of continuing infection. To determine if progeny virus from H4/CD4 was infectious, two tests of HIV-1 transmissibility were performed (Table 1). In the first experiment, cell-free HIV-1/N1T was harvested during the acute phase of infection of H4/CD4 or CEM cells and was used at the same dose to infect either H4/CD4 cells or CEM cells. H4/CD4 cells were highly susceptible to HIV-1 produced by T cells (Table 1), consistent with the results shown in Fig. 2. CEM cells were found to replicate N1T derived from H4/CD4 cells, but infection was slow (see Fig. 2 for comparison), suggesting that only a small fraction of the virus in the inoculum was infectious. In contrast, this virus was completely noninfectious in H4/CD4 cells (Table 1), a result consistent with a limited infectivity in the virus stock. To test progeny virus infectivity with greater sensitivity, N1T chronically infected H4/N1T-cl2 or CR10/N1T cells were treated with mitomycin and cocultivated with either uninfected H4/CD4 or CEM cells. Although chronically infected H4/N1T-cl2 cells produced some infectious virus, as indicated by its transmissibility to CEM cells, it was likely to be only a small proportion of the total virus produced, since it was unable to establish infection in uninfected H4/CD4 cells. This cannot be attributed to their resistance to infection, since H4/CD4 cells remained susceptible to infection by cocultivation with CR10/N1T cells (Table 1). We conclude that HIV-1 produced by H4/CD4 cells has significantly reduced infectivity and that this may contribute to the observed restricted infection in these cells.

Strain specificity of restricted HIV-1 replication in H4/CD4 cells. The studies described above used the uncloned virus isolate HIV-1/N1T for infection. To determine if the restricted viral replication observed is specific to N1T, we transfected H4/CD4 cells with viral DNA of 10 different molecular clones, including lymphotropic and macrophage-tropic strains, and monitored core antigen p24 production (Table 2). The clone Menv2, which does not synthesize envelope glycoprotein (72), was used in these experiments as a control nontransmissible virus. Peak p24 levels differed among different strains, with

lymphotropic clones being more productive. By 3 weeks after transfection, p24 levels had dropped from their peaks for all strains tested except the recombinant NL4-3 virus, which continued to spread infection (Table 2). In preliminary experiments, transfection of the molecular clone of Zairian isolate NDK (83) also resulted in unrestricted viral replication in H4/CD4 cells, similar to that observed with the NL4-3 clone (Table 2). The kinetics of p24 production after transfection of nine infectious viral DNAs which produced restricted infection in H4/CD4 cells were generally similar to those found after transfection of Menv2, an envelope mutant of HIV-1 (Table 2). These findings suggested that the restricted phenotype of HIV-1 replication in H4/CD4 cells shown in Fig. 2 for N1T virus was representative of the majority of HIV-1 strains tested, and they indicated that restricted infection may have been due to defective spread of progeny viruses produced in

TABLE 2. Replication of molecular clones of HIV-1 after transfection into H4/CD4 glioma cells^a

Viral clone	Reference	Tropism ^b	HIV-1 expression (ng of p24/10 ⁶ cells) at day posttransfection:		
			3	14	21
YU-2	50	MΦ	1.2	0.8	0.3
89.6	18	MΦ	0.1	0.2	0.1
NLHXADA-GP	96	MΦ	20	2.1	0.9
NLHXADA-PG	96	T	101	262	22
SF-2	75	T	0.1	0.01	0.01
HXBc2	47	T	8.4	3.1	1.5
N1T-A	74	T	12	33	30
NL4-3	1	T	134	1.5 × 10 ³	1.8 × 10 ⁴
Z6	84	T	22	25	1.9
Menv2	72	T	20	0.9	0.2

^a H4/CD4 cells were transfected with plasmid DNA, and the levels of cell-associated p24 core antigens were tested in cell lysates 3, 14, and 21 days after transfection as described in Materials and Methods. Each plasmid DNA was evaluated in this format at least three times in separate experiments.

^b MΦ, macrophages; T, T-cell lines.

FIG. 6. Analysis of intracellular and virion-associated viral proteins produced by HIV-1-infected glial and T-lymphoid cells. H4/CD4 and CEM cells were infected with HIV-1/N1T at an MOI of 1, cells were metabolically labeled either on day 3 or on day 7 after infection, and day 3 and day 7 cell extracts or day 3 sedimented culture supernatants were analyzed for HIV-1 antigens by immunoprecipitation with an AIDS patient serum, gel electrophoresis, and autoradiography as described in Materials and Methods. An autoradiogram from a representative experiment is shown. Major viral proteins are indicated at the left.

these cells. These results also suggested that NL4-3 (and NDK) differ from other HIV-1 strains in the progress of infection in H4/CD4 cells. Since two of the clones which replicated transiently in H4/CD4 cells, 89.6 and SF-2, have intact open reading frames for all of the auxiliary genes of HIV-1 (41, 59, 66), the data shown in Table 2 also indicate that restricted HIV-1 infection in H4/CD4 cannot be attributed to nonfunctional Vpu, Vpr, Vif, or Nef.

Expression of HIV-1 proteins in H4/CD4 cells. To determine if the reduced infectivity and spread of virus produced by H4/CD4 cells can be attributed to abnormalities in viral protein expression, we compared viral proteins in N1T-infected H4/CD4 and CEM cells by biosynthetic labeling and immunoprecipitation with AIDS serum (Fig. 6). Analysis of immunoprecipitates by SDS-PAGE showed that the cells expressed the major HIV-1 proteins similarly, except for high-molecular-mass proteins migrating with approximate molecular masses of 120 and 160 kDa and likely representing the envelope glycoproteins gp120 and gp160. CEM cells produced both p160 and p120 as expected; however, infected H4/CD4 cells produced only p160, with no p120 detectable. Virions sedimented from cell supernatants preserved these differences; virions from CEM cells carried only p120, and virions from H4/CD4 cells carried only a protein(s) with a molecular mass above 200 kDa (Fig. 6).

To confirm that the high-molecular-weight proteins that distinguish between HIV-1/N1T infection in CEM and H4/CD4 cells represent viral envelope glycoproteins, the previous experiment was repeated with the N1T-A virus cloned from N1T (74) and specific anti-gp120 as well as AIDS serum for immunoprecipitation. Eight percent polyacrylamide gels were run

for better resolution of high-molecular-weight proteins (Fig. 7A). Consistent with the results shown in Fig. 6, HIV-1-infected CEM and H4/CD4 cells differed in the expression of 120- and 160-kDa proteins which were precipitated by AIDS serum or by anti-gp120 antiserum. At the peak of HIV-1 infection, H4/CD4 cells contained no detectable gp120 envelope glycoprotein. These data suggest that H4/CD4 cells process N1T-A and N1T envelope glycoproteins aberrantly. To determine whether aberrant processing of gp160 is also characteristic of chronically infected cells, the H4/N1T-c12 clone, which synthesizes viral DNA and RNA (Fig. 5), was subjected to biosynthetic labeling and immunoprecipitation (Fig. 7B). The pattern of synthesis of the major viral proteins, with abnormal cleavage of gp160, was repeated in H4/N1T-c12. Thus, three kinds of infections produced similar results in our experiments: acute infection of H4/CD4 by the N1T strain (Fig. 6), acute infection by virus derived from a molecular clone (Fig. 7A), and chronic infection in an H4/N1T-c12 cell clone (Fig. 7B), all of which lead to the production of noninfectious progeny virus and the aberrant cleavage of gp160. Since similar results were obtained with bulk-infected cells and in a chronically infected cell clone, as well as with an uncloned viral strain and a molecular clone of the virus, it is unlikely that the aberrant processing of HIV-1 gp160 glycoprotein observed in these experiments is attributable to selection of a minority cell subpopulation or a minor variant of N1T virus.

To determine whether these processing abnormalities distinguish the fully productive infection by NL4-3 from the restricted infection by N1T, the experiment was repeated with H4/CD4 cells infected with either virus, and precipitation was performed with a specific anti-gp120 serum as well as AIDS sera (Fig. 8). Analysis of the immunoprecipitates of the cell lysates showed gp160 and gp120 in N1T-infected CEM cells and in NL4-3-infected H4/CD4 cells but only gp160 in the N1T-infected H4/CD4 cells. These findings indicate that H4/CD4 cells retain the capacity to cleave HIV-1 envelope glycoproteins, but carry out this function only in certain viral infections.

To determine whether the observed aberration in processing of the viral envelope glycoproteins in H4/CD4 resulted from altered proteolysis or from altered glycosylation, we repeated infections with N1T and subjected the immunoprecipitates to deglycosylation by digestion with three different glycosidases prior to electrophoresis. Figure 9A shows the results of analysis with PNGase F, which has a broad specificity, cleaving common N-linked oligosaccharides at the N-glycosidic bond (27). Figure 9B presents the results of a repeat experiment in which deglycosylation was performed with three glycosidases of different specificities: PNGase F; neuraminidase, which specifically cleaves neuraminic acid; and Endo H, which can cleave only oligomannose- and hybrid-type N-linked oligosaccharides (27). As shown in Fig. 9A, PNGase F-treated envelope proteins from CEM cells ran as two major bands with mobilities of about 95 and 55 kDa, respectively, corresponding to the deglycosylated forms of gp160 and gp120. N1T-infected H4/CD4 cells produced a deglycosylated envelope protein with a mobility similar to that of the larger protein produced by CEM cells, deglycosylated gp160. Thus, PNGase-treated gp160 proteins produced in infected H4/CD4 and CEM cells are indistinguishable from each other on SDS-PAGE gels. Comparison of treatments with three glycosidases in a repeat experiment (Fig. 9B) confirmed the similar patterns of deglycosylation of viral envelope proteins in infected H4/CD4 and CEM cells: as in the first experiment, PNGase F treatment converted gp160 produced in both cells to proteins migrating at 95 kDa; neuraminidase had no effect on protein mobility in either system,

FIG. 7. Envelope glycoprotein synthesis in acutely HIV-1-infected H4/CD4 cells and in a chronically infected cell clone, H4/N1T-c12. CEM and H4/CD4 cells infected with HIV-1/N1T at an MOI of 1 (A) and the H4/N1T-c12 cell clone (B) were metabolically labeled, and cell extracts were tested for the presence of HIV-1 glycoproteins by immunoprecipitation with anti-gp120 antiserum, AIDS patient serum, or HIV-1-negative serum as described in Materials and Methods. Protein molecular mass markers are shown at the left.

while Endo H treatment generated a major species of deglycosylated protein which had an electrophoretic mobility of about 90 kDa, regardless of whether it was produced in H4/CD4 or CEM cells (Fig. 9B). These results suggest that the intracellular compartmentalizations of viral envelope proteins during glycosylation in H4/CD4 and CEM cells are similar and that the major difference between the production of most HIV-1 species by H4/CD4 cells and that by CEM cells resides in the proteolytic cleavage of gp160.

Sequence of the envelope cleavage site in N1T-A. To determine if N1T-A and NL4-3 envelope proteins differ in the region encompassing the cleavage site between HIV-1 gp120 and gp41 (25, 54), we sequenced the envelope region of N1T-A and compared it with that of NL4-3 (Fig. 10). The critical region containing both potential cleavage sites (underlined in Fig. 10) is shared by these two viruses, including the conserved arginine-X-lysine-arginine, which is present in the envelope proteins of most lentiviruses (54). The W-to-V exchange in position 450 (Fig. 10) is included within the N terminus of gp41, and it likely does not play a role in proteolytic cleavage between gp120 and gp41 (25, 54). We conclude that the inability of H4/CD4 to process N1T-A envelope cannot be attributed to its primary structure containing the two known proteolytic cleavage sites.

DISCUSSION

The development of a glial cell line highly susceptible to HIV-1 infection enabled us to define a new, naturally occurring restriction in the virus life cycle: during infection, H4/CD4 cells synthesize viral DNA, RNA, and protein normally, but generally fail to cleave gp160 into the mature gp120 and gp41 required for viral transmission. Since this proteolysis is carried out by cellular enzymes (31, 43, 58) and proteolysis was normal in the case of one viral clone tested, H4/CD4 cells may be

FIG. 8. Comparison of envelope glycoprotein expression in H4/CD4 cells infected with N1T-A and NL4-3 viral clones. CEM and H4/CD4 cells were infected with HIV-1/N1T-A or NL4-3 at an MOI of 1 and were subjected to metabolic labeling and immunoprecipitation with anti-gp120 antiserum 3 days after infection as described in Materials and Methods.

FIG. 9. Comparison of glycosylated and deglycosylated envelope glycoproteins in HIV-1-infected H4/CD4 and CEM cells. Cells were infected with HIV-1/N1T-A at an MOI of 1, and 3 days after infection they were subjected to metabolic labeling, deglycosylation, and immunoprecipitation with HIV-1 envelope protein antisera as described in Materials and Methods. (A) Analysis with PNGase F after immunoprecipitation with rabbit gp120 antiserum. (B) Analysis with PNGase F, neuraminidase, and Endo H in a repeat experiment in which goat gp160 and gp120 were used for immunoprecipitation. The autoradiogram image from this experiment was captured by video camera and processed with the Paintshop Pro Program.

inferred to regulate the activity or synthesis of specific proteases or compartmentalization of viral proteins during HIV-1 infection.

In previous studies, several events in the HIV-1 life cycle have been identified which lead to restricted infection in glial cells. Stable expression of transduced CD4 in the U87MG glioma cell line failed to confer susceptibility to syncytium formation with HIV-1-producing cells or virus infection (12, 15), suggesting that HIV-1 is unable to fuse with certain CD4-bearing glial cells. Studies with another transformed glial cell line, U373MG, were consistent with this result; they indicated that HIV-1-infected CD4-bearing glial cells were unable to synthesize viral DNA detectable by PCR (40). It is notable that in one study, cells insensitive to HIV-1 fusion or production were susceptible to both HIV-2 and simian immunodeficiency virus, suggesting that some strain differences may contribute to the observed restricted HIV-1 host range (15). The failure of HIV-1 entry or internalization in these stably CD4-transduced glial cells has been attributed to the absence of cellular cofactors required for the CD4-gp120/gp41-mediated fusion (12, 15, 40). In contrast, the display of CD4 on glial H4 cell membranes, like that on HeLa cells (40, 53), was sufficient to mediate efficient HIV-1 fusion and entry as indicated by the synthesis of high levels of viral DNA within hours after infection (Fig. 3 and 4). Thus, the presumptive absence of cellular cofactors required for HIV-1 entry is not a general property of CNS-derived cells. It will be of interest to determine whether H4/CD4 cells display one or more of the recently identified fusion receptors for T-cell-line-tropic and macrophage-tropic HIV-1 strains (2, 14, 20, 22, 23, 30).

After acquisition of viral DNA by transfection (77, 85, 88, 89), or as shown here by infection, glial cells also restrict HIV-1 production. This is particularly prominent in the system described here because of the initial high levels of HIV-1 production, approximating those seen in HIV-1-infected T cells (Fig. 2). This result is consistent with the findings of our previous study, in which we have shown that glial cells stably carrying transduced HIV-1 DNA and producing virus at high levels also cease virus production upon prolonged culture but can be induced to moderate expression by sodium butyrate (77). In this and in a previous study, all major forms of viral RNA diminished over time in concert with reduction in viral p24 production (Fig. 4 and reference 77), suggesting that the cellular control of HIV-1 expression in this system did not involve differential regulation of viral transcription. In contrast, the establishment of viral persistence in human fetal

astrocytes transfected with NL4-3 viral DNA was accompanied by the reduction in the expression of viral 9- and 4-kb mRNAs, but not of the 2-kb mRNAs encoding primarily Tat, Nef, and Rev proteins (88). Induction of these cells by tumor necrosis factor alpha increased both structural and regulatory gene expression, but Nef-specific mRNA and protein predominated (88). Other investigators have shown directly that Rev function is impaired in persistently infected glial cell lines compared with T-cell lines (63). Together, these studies point to viral entry, RNA production, and Rev-dependent posttranscriptional regulation of HIV-1 expression as multiple regulatable junctures during early stages of HIV-1 replication in neural cells.

The findings presented here address a later, posttranslational stage of regulation of HIV-1 infection in glial cells. H4/CD4 cells produce viral DNA, RNA, and proteins, indicating that virally encoded functions required for HIV-1 infections were normal. Full-length viral DNA was produced and was present in a high-molecular-weight form, presumably integrated, and this presumption was confirmed by examination of a chronically infected H4/CD4 cell clone (Fig. 3 and 5). The three major transcripts of viral RNA were synthesized in ratios similar to those in T cells, the major viral proteins were also synthesized, and p24-containing viral particles were released extracellularly (Fig. 4, 6, and 7). Nonetheless, the levels of all viral products declined in H4/CD4 cells after an early peak, but they continued to increase in CEM cells. We have determined that this extinction of virus infection is due to the inability of progeny virus to spread infection. Both cell-free and cell-mediated virus transmissions were impaired (Table 1). We attribute the defective infectivity of virus produced in H4/CD4 cells to a defect in proteolytic processing of viral precursor envelope glycoprotein gp160, because H4/CD4 cells acutely infected with either the N1T isolate or the N1T-A molecular clone contained gp160 but not gp120 (Fig. 6 to 8). The absence

FIG. 10. Comparison of envelope protein sequences of N1T-A and NL4-3. The region of NL4-3 containing the potential cleavage sites between gp120 and gp41 was aligned to the corresponding region from N1T-A. The sites designated as primary (1°) and secondary (2°) proteolytic cleavage sites (25) are indicated (underlined) as is the site of gp120/gp41 cleavage (arrow).

of gp120 could not be attributed to defective glycosylation of the precursor envelope protein, because deglycosylation experiments with glycosidases of different specificities did not reveal significant differences in electrophoretic mobilities of precursor envelope proteins made in H4/CD4 and CEM cells (Fig. 9). Blocking of gp160 cleavage by mutations in the proteolytic cleavage site (25, 54, 99, 100) or in T cells unable to process gp160 (26) resulted in the production of noninfectious HIV-1. Although there is controversy whether uncleaved gp160 can be incorporated into virions (25, 100), it is accepted that uncleaved gp160 cannot mediate fusion with CD4-positive cells (25, 26, 99, 100). We conclude that the failure to cleave gp160 limits HIV-1 infection in H4/CD4 cells to one or few rounds of replication, similar to the restricted viral spread observed after transfection of HIV-1 *env*-negative mutant DNA (Table 2). Since the N1T virus produced in H4/CD4 could eventually be rescued by culture in T cells (Table 1), the blocking of gp160 proteolytic processing in these glial cells is not absolute, and it may differ in its extent for different viral clones (Table 2). It has been reported that during infection of T lymphocytes, only a small fraction of gp160 is cleaved to mature gp120 and gp41 (99), and H4/CD4 may reduce this fraction still further, to a level which does not permit spread of infection. Whatever the mechanism, the defective gp160 processing during N1T infection is a general feature of H4/CD4 cells, because it was observed both during acute infection of bulk cell populations and in a chronically infected H4/CD4 cell clone.

The restricted HIV-1 infection in H4/CD4 cells was observed with eight of nine infectious HIV-1 clones tested. One clone, NL4-3, was able to produce efficiently spreading infection, and NL4-3-infected H4/CD4 cells expressed both gp160 and gp120, suggesting that gp160 cleavage occurred normally (Table 1 and Fig. 8). In preliminary experiments, we found that the molecular clone of an African HIV-1 isolate, NDK (83), also exhibited an NL4-3-like pattern of replication in H4/CD4 cells. This indicates that the absence of cleavage of gp160 in H4/CD4 is virus strain specific, and it is common to the majority of the HIV-1 strains tested. It is puzzling that H4/CD4 cells discriminate between highly homologous polypeptides for cleavage (Fig. 10). Even NLHXADA-PG, which is essentially NL4-3 with a homologous HXB-2 replacement and a small heterologous ADA replacement (96), replicated only transiently in H4/CD4. The cellular proteases which catalyze the gp160 cleavage have not been fully identified (31, 43, 58, 65), and H4/CD4 cells may express a rare enzyme which can recognize a presently unknown cleavage motif in NL4-3 (and NDK) envelope proteins. Alternatively, NL4-3 and NDK may differ from other HIV-1 clones tested here in the intracellular trafficking of precursor envelope protein such that greater fractions of gp160 are processed rather than being degraded in lysosomes (99). The means to control the efficiency of envelope processing is currently under investigation.

We have described a novel, posttranslational mechanism responsible for a virus strain-specific restriction of HIV-1 infection in CD4-expressing human glial cells in vitro, namely a defective proteolytic processing of precursor envelope glycoproteins. A similar mechanism was found to be responsible for the restricted infection of microglial cells by a highly neurovirulent murine retrovirus, FrCas^E (52). Our data add to the growing body of evidence indicating that human neural cells control HIV-1 infection by several different mechanisms. It is unclear at present which specific mechanism(s) is responsible for the observed restricted HIV-1 infection of astrocytes (73, 87) and possibly other neural cells (64) in vivo. Our attempts at biochemical analysis of envelope glycoproteins in HIV-1-infected CD4-negative U-257 glial cells and primary astrocytes

were hampered by inefficient expression of viral proteins in these cells (92a). Use of H4/CD4 cells, which as shown here are permissive for efficient primary infection by HIV-1, will permit detailed molecular studies of the control of HIV-1 protein expression in nervous system-derived cells and may lead to the development of novel ways to control HIV-1 replication.

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