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Persistent Productive Infection of Human Glial Cells by Human Immunodeficiency Virus (HIV) and by Infectious Molecular Clones of HIV

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The nature of the interaction between human immunodeficiency virus (HIV) and human cells of astrocytic origin was studied in vitro with cultured glial cells and intact HIV or infectious molecular clones of the virus. Infection of glial cells with intact HIV was characterized by low-level expression of viral transcripts as detected by Northern blotting and in situ hybridization (<10 copies of HIV RNA per cell), transient virus replication, absence of viral antigens detectable by immunofluorescence, and complete lack of cytopathic effects. However, the HIV-infected glial cells persistently expressed HIV *tat*III gene activity as detected by a chloramphenicol acetyltransferase assay, and HIV transcripts could be detected by in situ hybridization in 20 to 30% of cells up to 4 months after infection, suggesting that the lack of cytopathicity in HIV-exposed cells was not due to transient viral infection. To evaluate whether increased expression and replication of HIV in glial cells would have any effect on cell growth and viability, we established HIV-positive glial cell lines by cotransfection of cells with infectious molecular clones of HIV DNA and a selectable marker gene. Three clones were isolated which produced high levels of viral particles, were strongly positive for HIV antigens by immunofluorescence, and contained >1,000 copies of HIV RNA per cell. These cell lines showed no cytopathic changes (lysis, fusion), and their growth kinetics were similar to HIV$^-$ controls, but significant morphological changes were detected (cytoplasmic swelling; increased numbers of rounded, presumably detaching cells). Our results show that astrocytic cells can support a persistent, replicative HIV infection with limited pathogenic effects.

The acquired immunodeficiency syndrome (AIDS) is associated with immunological, neurological, and gastrointestinal disorders which are characterized by opportunistic infections, malignancies, chronic diarrhea, and progressive dementia (15, 31). Although the human immunodeficiency virus (HIV) is believed to be responsible for the severe immune impairment of AIDS patients, largely as a result of its interaction with receptor molecule CD4 (T4)-positive hematopoietic cells (T lymphocytes [25], monocyte-macrophages [19]), the underlying causes of the other manifestations of AIDS, such as gastrointestinal and central nervous system disorders, remain unclear. It has been hypothesized that HIV is involved in the pathogenesis of the AIDS dementia complex on the basis of the detection of HIV in the brain tissue of neurosymptomatic AIDS patients (33, 40) and by analogy to neurotropic lentiviruses, such as visna virus, which share striking sequence homology to HIV (34). It is unclear, however, precisely how HIV might induce brain damage, since little is known of the susceptibility to, or effects of, HIV infection in human glial cells.

Our preliminary studies (11) have demonstrated that HIV can indeed directly infect cultured human glial cells, a finding that has been confirmed recently by other workers (7, 8, 26). However, the interaction of HIV with brain-derived cells appeared to be markedly different from that observed in other virus host cells. HIV replication was minimal, as indicated by the lack of significant levels of reverse transcriptase (RT) activity in culture supernatants, the failure to obtain convincing Southern blots or electron micrographs, and the inability to detect viral antigens in infected cells by indirect immunofluorescence staining (7, 8, 11, 26). In addition, no cytopathic effects were observed after HIV infection, in contrast to the massive lytic effects observed in human T lymphocytes (25).

The underlying cause for the observed lack of viral cytopathicity in cultured glial cells is unclear, and previous studies have failed to convincingly demonstrate whether the infection of human glial cells was a transient phenomenon. The present work was undertaken to investigate this question and to study the molecular basis for the low-level expression of HIV in glial cells exposed to intact virus. Furthermore, highly HIV-positive glial cell lines were established to determine whether such cells would undergo virally induced cytopathic effects, alterations in growth kinetics, or changes in cellular gene expression.

Our results show that cultured human glial cells can be persistently and productively infected with HIV. Interestingly, the infected cells maintain normal growth characteristics but undergo significant morphological changes. These results indicate that HIV infection of astrocytes in the brain may contribute to the pathogenesis of the AIDS dementia complex.

MATERIALS AND METHODS

Cells and viruses. Human glial cell lines U-251MG (H80) (4) and U-373MG (38) were obtained from D. D. Bigner and the American Type Culture Collection (Rockville, Md.),

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respectively, and were cultivated in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The CD4-positive T-cell line CEM (16) was received from L. Montagnier, and the HIV lysis-resistant subclone of CEM, CR10, was established in our laboratory (6). The suspension cultures were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum. The N1T isolate of HIV (32) was propagated in CEM or CR10 cells. Glial cells were infected with cell-free, virus-containing supernatants as described previously (11).

Plasmids. Plasmid pSV2neo (37) was obtained from Bethesda Research Laboratories (Gaithersburg, Md.). The murine glial fibrillary acidic protein (GFAP) cDNA (28) was donated by M. Shelanski and subcloned into the plasmid pGEM4 (Promega Biotec, Inc., Madison, Wis.) to allow the synthesis of both sense- and antisense-orientation RNAs. The HIV TAT region probe, pTBtat, was prepared similarly with the 0.7-kilobase (kb) *Eco*RI-*Kpn*I fragment of HIV N1T proviral DNA (32). The infectious HIV DNA clone pN1T-E2 was prepared as described elsewhere (K. Sakai, S. Dewhurst, C. Meier, and D. J. Volsky, submitted for publication). The nearly full-length HIV DNA probe used for Southern blot analysis was prepared as an 8.9-kb *Sac*I-*Sac*I fragment from the HIV N1G-G clone (32). Plasmid X-TAT was donated by S. Rhode, and plasmid RSV CAT (20) was obtained from the American Type Culture Collection.

Chemicals and radiochemicals. Photobiotin was purchased from Bresa Ltd. (Adelaide, South Australia). Fluorochrome-conjugated or native streptavidin and RNases A and T1 were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Nytran membrane was obtained from Schleicher & Schuell Co. (Keene, N.H.), G418 was from GIBCO Laboratories (Grand Island, N.Y.), and fluorescein isothiocyanate-conjugated anti-human immunoglobulin G was from Tago Immunochemicals (Burlingame, Calif.). All radiochemicals were obtained from New England Nuclear Corp. (Boston, Mass.), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

In situ hybridization. Cell smears were fixed for 20 min at room temperature in 75% ethanol–20% acetic acid and dehydrated for 5 min in 95% ethanol. Slides were rehydrated in phosphate-buffered saline (Ca^{2+} and Mg^{2+} free) and taken through a modified (30) prehybridization procedure (5) before overnight incubation with a Photobiotin-labeled (17) antisense single-stranded RNA probe. The probe consisted of either pTBtat (for HIV) or pGFAP (for GFAP) in the hybridization cocktail as described previously (17). The antisense RNA probe was transcribed by using the T7 promoter of the *Hin*dIII-linearized transcription plasmid template. After hybridization, slides were washed five times at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% Triton X-100 for 15 min. Slides were then treated with RNase (10), washed twice as described above, and then washed twice more at 50°C in 0.1× SSC–0.1% Triton X-100 for 30 min each time. Hybrids were detected by incubation of the slides with streptavidin-conjugated rhodamine (for HIV) or streptavidin-conjugated fluorescein isothiocyanate (for GFAP) according to the manufacturer's instructions. Photographs were taken on a Nikon microscope with Kodak Ektachrome PS 800/1600 film push processed at 1600 ASA. Exposure times were approximately 25 s. All slides were photographed with the same exposure to allow direct comparison of the relative levels of fluorescence.

CAT assay. Glial cells were transfected with chloramphenicol acetyltransferase (CAT) plasmid constructs by the calcium phosphate (21) method with carrier DNA and glycerol shock to facilitate entry of DNA into cells. After incubation of cells for 48 h, cell lysates were prepared and assayed for CAT activity as described previously (20). The CAT reaction mixtures contained 0.02 ml of cell lysate, 0.07 ml of 0.25 M Tris hydrochloride (pH 7.8), 0.02 ml of 4 mM acetyl coenzyme A, 0.0005 ml of [^{14}C]chloramphenicol (specific activity, 50 mCi/mmol), and 0.035 ml of H_2O. The reaction times were varied to insure that the rate of product formation was in the linear range. The reactions were extracted with ethyl acetate and resolved by thin-layer chromatography. Chromatography plates were then sprayed with En^{3}Hance (New England Nuclear) and exposed overnight to Kodak XAR-2 film with intensifying screens.

Preparation of cellular DNA and RNA. Total cellular RNA was prepared by the CsCl-guanidinium thiocyanate method (9). Cellular RNA, which pelleted through the CsCl cushion, was washed twice with 200 μl of absolute alcohol at room temperature, suspended in 400 μl of diethylpyrocarbonate-treated H_2O, and precipitated with 2 volumes of absolute ethanol at −20°C in the presence of 0.3 M sodium acetate (pH 5.2). Cellular DNA remaining at the CsCl-guanidinium thiocyanate interface was diluted with 2 volumes of Tris-EDTA, extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with an equal volume of chloroform-isoamyl alcohol, and precipitated with 5 volumes of 80% alcohol at −20°C.

Southern blot analysis. Restriction endonuclease-digested DNA (10 μg) was electrophoresed in 0.8% Tris borate (pH 8)–agarose gels and transferred to Nytran by the procedure of Southern (36). Filters were prehybridized and hybridized at 42°C in a solution containing 5× SSC, 50% formamide, 1% sodium dodecyl sulfate, 3× Denhardt reagent, 100 μg of denatured salmon sperm DNA per ml, and 5 mM EDTA (pH 8). Hybridization continued for 24 h in hybridization cocktail (see above) supplemented with 1×10^6 to 2×10^6 cpm of ^{32}P-labeled DNA probe (specific activity, 2×10^8 to 5×10^8 cpm/μg, as described above) per ml. Hybridized filters were washed with brisk agitation in three changes of 0.2× SSC–0.1% sodium dodecyl sulfate at 55 to 60°C. The washed filters were blotted dry and subjected to autoradiography with Kodak XAR-2 film with intensifying screens at −80°C. Bacteriophage λ DNA digested with *Hin*dIII and end labeled with ^{32}P by using DNA polymerase I large fragment was used for size markers.

Transfection-selection experiments. Glial cells were transfected with approximately 5 μg of DNA per 10^6 cells (10:1 ratio of HIV DNA to pSV2neo DNA) by the calcium phosphate method (21). Two weeks thereafter, selection was initiated by using culture medium supplemented with 50 μg of G418 per ml. Drug levels were gradually increased weekly to 400 μg/ml until outgrowth of G418-resistant cell colonies became apparent (after ca. 8 weeks). Colonies were isolated, subcloned into 24-well plates in the presence of 1.6 mg of G418 per ml, and tested for the expression of HIV antigens by immunofluorescence.

Immunofluorescence assays. Cells were washed in phosphate buffered saline, spotted on a slide, dried, and fixed in acetone at −20°C for 15 min. The fixed cells were reacted with an HIV-reactive, human T-lymphotropic virus type I-nonreactive serum from a hemophilia patient (D-771) at a 1:20 dilution. This serum contains antibodies to all of the major viral polypeptides, as determined by Western blot (immunoblot) analysis. After a 30-min incubation at 37°C, the cells were reacted with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G and read under an EPI fluorescent microscope (American Optical, Buffalo, N.Y.).

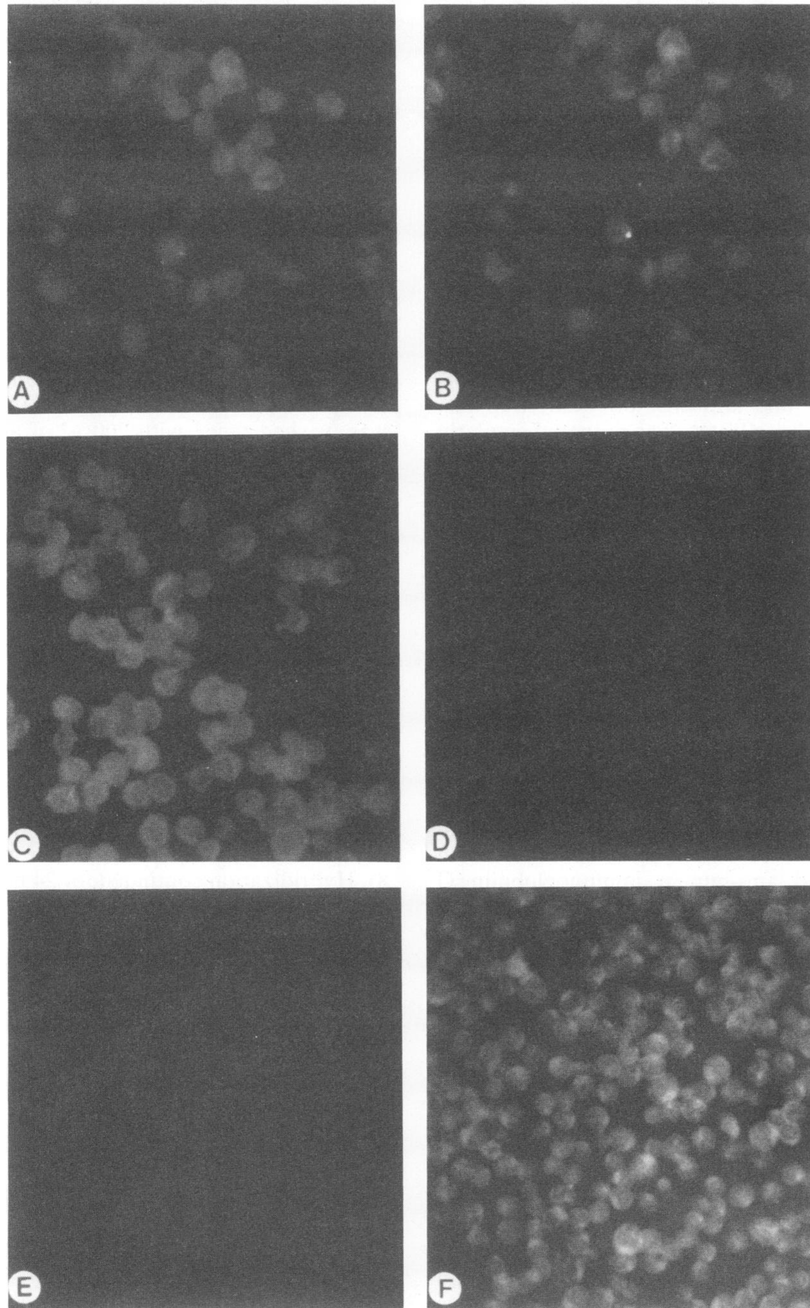

FIG. 1. Analysis of HIV-infected cells by in situ hybridization. In situ hybridization analysis of fixed cell smears from cultures of U-373MG (panels A through D) or CR10 (panels E and F) (6) cells at 3 (panels A and B) or 14 days (panels C, D, and F) after exposure to cell-free culture supernatants containing the N1T isolate of HIV (32). Photobiotinylated probes used were either antisense orientation HIV RNA (panels A, C, E, and F), antisense GFAP RNA (panel B), or sense-strand HIV RNA probe (panel D). In the case of HIV, streptavidin-rhodamine was used as a reporter molecule, whereas for GFAP, fluorescein isothiocyanate-conjugated streptavidin was chosen to allow simultaneous detection of both mRNAs. Magnification, ×250.

RT assays. Poly(rA)-dependent DNA polymerase activity (RT) was measured in 0.05 ml of 50 mM Tris hydrochloride (pH 7.5)–5 mM dithiothreitol–100 mM KCl–10 mM $MgCl_2$–5 μM [^{3}H]dTTP–0.1% Triton X-100 containing 2 μg of poly(A), 0.4 μg of oligo $(dT)_{12-18}$, and an appropriate amount of virus preparation. The reaction mixture was incubated at 37°C for 1 h, and then the [^{3}H]dTMP incorporated into polymers was precipitated with 10% trichloroacetic acid, collected on filters, and counted in a liquid scintillation counter.

Quantitation of HIV RNA in infected cells. Quantitation of RNA in cells was achieved essentially as described (31a). Briefly, cells were solubilized in 4 M guanidinium isothiocyanate, and 12.5-μl samples containing 10^5 cells were used for direct hybridization at room temperature with increasing quantities of ^{32}P-labeled HIV RNA probe generated in an antisense orientation from the plasmid pGAP (11). Unhybridized probe was digested with RNases A and T1, RNA-RNA hybrids were collected on nitrocellulose filters,

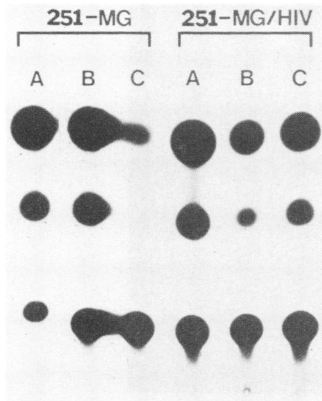

FIG. 2. Identification of HIV in long-term cultures of infected U-251MG cells. Uninfected and virus-infected U-251MG cultures (at 4 weeks after exposure to HIV) were transfected with the plasmids containing the bacterial *cat* gene linked to the RSV long terminal repeat (plasmid RSV *cat*, lanes A) or the HIV long terminal repeat (plasmid HIV LTR *cat*, lanes C). As a positive control, the HIV long terminal repeat *cat* was cotransfected into cells together with an expression vector which produces *tat*III protein (plasmid X-TAT, lanes B). CAT activity was assayed in one fifth of the cell extract 48 h after transfection.

and bound radioactivity was counted in a scintillation counter. Maximum hybridization values on the saturation curve were extrapolated to the ordinate to yield a saturation value that was used to calculate the average number of available HIV RNA targets per cell.

Determination of cell growth kinetics. Cells were removed by trypsinization, reseeded in 24-well plates at 10^4 cells per well in triplicate for each test point, and cultured under standard conditions. At 24 h before each test point, 10^5 cpm of [^{3}H]thymidine (specific activity, 2.0 mCi/mmol) was added to each well, and incubation was continued for 24 h. At the designated time points, cells were washed three times in phosphate-buffered saline, and plates were frozen at −80°C. At the end of the experiment, the plates were thawed, cells were disrupted with 1 ml of cold water per well, and the lysates were tested for protein by the method of Lowry et al. (29). Incorporation of [^{3}H]thymidine into total cellular DNA was determined by filtration of lysates through a 0.45-μm filter, followed by extensive washing and measurement of bound radioactivity in a scintillation counter.

RESULTS

Infection of GFAP-positive astrocytes by intact HIV. The human glioma-derived cell lines U-373MG and U-251MG, which we have previously shown to express the astrocytic marker GFAP as well as the HIV receptor molecule CD4 (12), were exposed to HIV-containing culture supernatants from CR10/N1T producer cells. At various time points, the cultures were assayed for the presence of HIV. In confirmation of previous observations (7, 8, 11), HIV-specific gene transcripts were demonstrated in approximately 30% of the glial cells at 3 days after infection by using in situ hybridization (Fig. 1A) and in chronically infected T cells (CR10/N1T, Fig. 1F) but were absent from uninfected cells (Fig. 1E). No hybridization was observed when HIV RNA probes in a sense orientation were used, confirming the specificity of this detection method (Fig. 1D). By using a double detection technique with HIV and GFAP, HIV transcripts were localized to GFAP-expressing (and therefore astrocytic [13]) cells (Fig. 1A and B), unequivocally demonstrating that astrocytic cells can be infected by HIV, as we and others have previously suggested (7, 11).

Persistence of HIV in human glial cells. Despite the presence of HIV-derived RNA transcripts in a high proportion of cells in infected glial cell cultures, viral antigens could not be detected by immunofluorescence staining, culture supernatants were negative for reverse transcriptase activity, and convincing Southern blots for the presence of HIV DNA could not be obtained. This was in agreement with other published data (7, 8, 11, 26) and raised the question as to whether the HIV infection of cultured human glial cells was transient or persistent in nature.

In situ hybridization analysis of long-term glial cell cultures indicated that HIV gene activity persisted for between 14 days (Fig. 1C) and 4 months after infection (data not shown). The CAT assay confirmed this observation. Functional HIV *tat*III gene product was present in U-251MG cells at 1 month after exposure to HIV but absent from unexposed cultures (Fig. 2, lanes C). The functionality of the HIV *trans*-activational pathway in the U-251MG glioma cell line was demonstrated by the high levels of CAT enzyme activity detected after cotransfection of cells with plasmids encoding the *tat*III gene product (plasmid X-TAT) and an HIV long terminal repeat-linked CAT gene (plasmid HIV LTR CAT) (Fig. 2, lanes B). The levels of CAT activity in these cotransfected cultures approximated those observed in cultures that were transfected with the CAT gene fused to a Rous sarcoma virus long terminal repeat (plasmid RSV CAT) (Fig. 2, lanes A).

Lack of cytopathic effects in HIV-infected glial cells. Figures 3A and B illustrate the morphology of uninfected and HIV-infected U-251MG cultures, respectively. Neither short-term (21 days; Fig. 3B) nor long-term (4 months; data not shown) cultures of HIV-infected cells exhibited any of the typical cytopathic effects associated with HIV infection of human T lymphocytes, i.e., lysis and cell fusion.

Establishment of highly HIV-positive glial cell lines. The lack of observable cytopathic effects in HIV-infected glial cell cultures could have been a consequence of the inefficient entry of HIV into the cells, due to the low level of virus receptor molecules (12). To facilitate viral penetration and selection of virus-positive cells, glial cells were cotransfected with an infectious recombinant HIV clone, N1T-E (Fig. 4), and plasmid pSV2neo, which encodes the selectable gene for aminoglycoside transferase (*neo*). After introduction of exogenous DNA into U-251MG cells, clones were selected in the presence of G418 and screened for HIV antigen expression by immunofluorescence staining. Three HIV-positive cell lines were established at 8 weeks after transfection, and a number of G418-resistant, HIV-negative clones were also derived after transfection of cells with pSV2neo alone. The principal characteristics of the various cell lines obtained by drug selection are summarized in Table 1. The three HIV-positive cell lines were all of astrocytic origin, as judged by immunofluorescence staining with monoclonal antibodies reactive with the astrocyte-specific antigen GFAP. In addition, these lines produced high levels of infectious HIV during 5 months of continuous culture, as determined by RT activity in culture supernatants, electron microscopy, and virus rescue experiments with T4-positive human T lymphocytes (Table 1).

Lack of viral cytopathicity in HIV-releasing astrocytic lines. Although the three HIV-antigen positive 251-MG subclones were persistently and productively infected by HIV, they did not appear to undergo gross lysis or syncytium formation.

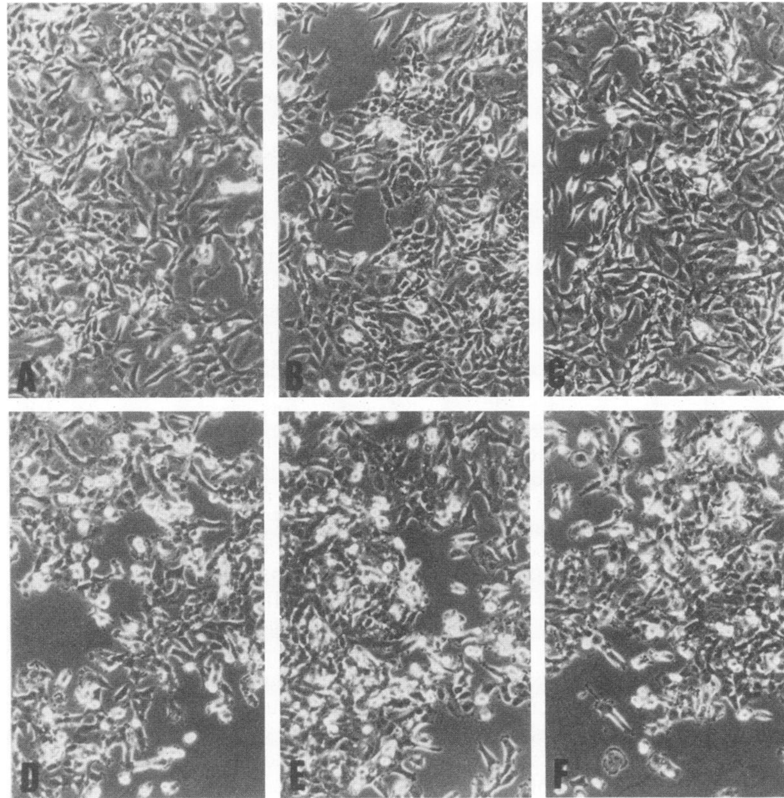

FIG. 3. Photomicrographs of HIV-positive and -negative U-251MG sublines. Light microscopic appearance of U-251MG cells before (panel A) and 3 weeks after (panel B) direct infection with HIV. Also shown are the G418-resistant, HIV-positive lines 251-MG/E_a (panel D), 251-MG/E_b (panel E), and 251-MG/E_c (panel F) as well as the G418-resistant, HIV-negative control 251-MG/Neo (panel C). Magnification, ×50.

However, examination of these cultures under light microscopy revealed that significant alterations had occurred in the morphology of the cells. Figure 3C illustrates the appearance of the HIV-negative, G418-resistant control clone 251-MG/neo, whereas Fig. 3D, E, and F show the HIV-positive subclones 251-MG/E_a, 251-MG/E_b, and 251-MG/E_c, respectively. Significant differences were observed between HIV-positive and HIV-negative cells in terms of both cytoplasmic swelling in cells and the proportion of rounded, presumably detaching, cells. These differences were most pronounced in the 251-MG/E_a subclone compared with the other HIV-positive lines (Fig. 3).

Growth kinetics of persistently infected HIV-producing glial cell lines. Since there was a discernable morphological change in HIV-positive cultures as compared with controls, the growth kinetics of two of these 251MG cell lines (251-MG/E_a and 251-MG/E_b) were compared with that of an HIV-negative, G418-resistant control (251-MG/neo). 251-MG/E_b exhibited kinetics of cell growth and DNA synthesis identical to those of the HIV-negative 251-MG/neo line (Table 2). The 251-MG/E_a cell line exhibited slower kinetics of cell growth and DNA synthesis during the first 3 days of culture but also became confluent within 5 to 7 days after subculture. All of the clones remained fully viable regardless of the extent of HIV replication (data not shown).

Differences in cell morphology correlate with levels of intracellular HIV RNA. The number of HIV RNAs per cell was calculated on the basis of hybridization saturation curves (Fig. 5). The HIV RNA load per cell varied from approximately 10,000 in lytically infected CEM cells (data

TABLE 1. Characteristics of G418-resistant U-251MG sublines obtained after transfection experiments

Cell line	% GFAP-positive cells[a]	% HIV antigen-positive cells[b]	HIV RNA copies/cell[c]	RT activity[d]	Virus release[e]
251-MG/E_a	100	>80	1,900	271×10^3	+
251-MG/E_b	100	60	1,300	191×10^3	+
251-MG/E_c	100	50	1,200	106×10^3	+
251-MG/Neo	100	0	2	0.8×10^3	−
CR10/N1T	0	90	3,000	361×10^3	+++
CEM/N1T	0	90	10,000	$>500 \times 10^3$	+++

[a] Determined by immunocytochemistry with monoclonal antibodies as described previously (12).
[b] Determined by indirect immunofluorescence as described in Materials and Methods.
[c] Determined by chaotropic hybridization as described in Materials and Methods.
[d] Mean of four observations over a period of 1 month.
[e] Determined by electron microscopy and rescue experiments.

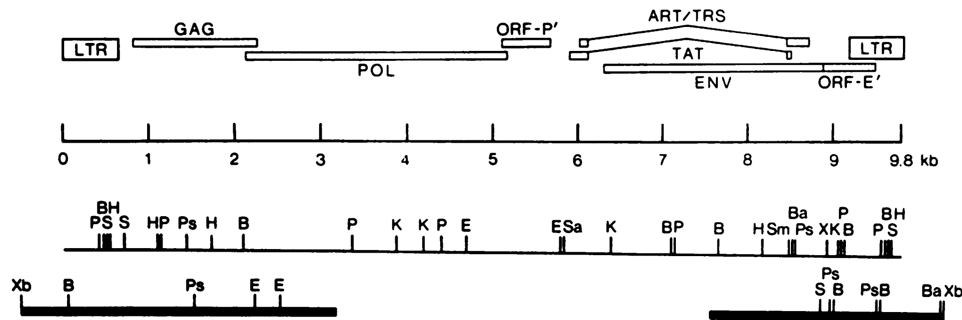

FIG. 4. Map of biologically active HIV DNA clone N1T-E. The N1T-E clone contains a full-length HIV provirus (—) flanked by cellular sequences (▬) cloned into pUC-18. Restriction enzyme cleavage sites: Ba, *Bam*HI; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Ps, *Pst*I; P, *Pvu*II; S, *Sac*I; Sa, *Sal*I; Xb, *Xba*I; X, *Xho*I. LTR, Long terminal repeat.

not shown) to 3,000 for non-cytopathically infected CR10/N1T cells and 1,900, 1,300, and 1,200 copies per cell in 251-MG/E_a, 251-MG/E_b, and 251-MG/E_c cells, respectively (Fig. 5). All major viral transcripts were detected on Northern blot analysis of cellular RNAs (data not shown), and the control 251-MG/neo subclone was negative for HIV RNA (background radioactivity only). Interestingly, the 251-MG/E_a subclone contained the highest level of HIV RNA per cell and also exhibited the most significant alterations in cellular morphology of all of the HIV-positive lines. This result was even more striking in light of the differences in HIV load in lytically infected T-lymphocytic cells (CEM line) as compared with the non-cytopathically infected CEM subclone CR10/N1T.

Status of HIV DNA in virus-positive 251-MG sublines. Genomic DNA was isolated from the various 251-MG sublines, digested with appropriate restriction endonucleases, and analyzed by Southern blot hybridization with a nearly full-length HIV DNA probe. An example of such analysis, after digestion with either *Eco*RI or *Pst*I, is shown in Fig. 6. HIV-infected CEM cells served as a positive control, containing approximately 20 copies of HIV DNA per cell (our unpublished observations). *Eco*RI digestion of DNA from HIV-infected CEM cells typically produced a fragment of 8.8 kb in size, which corresponds to unintegrated, circular HIV DNA as well as smaller fragments of 4.7, 4.0, and 1.1 kb. In contrast, *Eco*RI and *Pst*I digests of DNA from 251-MG sublines contained only those fragments that derive from the HIV provirus contained in the plasmid pN1T-E (Fig. 4). *Eco*RI digestion resulted in bands of 6.8 and 5.5 kb, and *Pst*I digestion resulted in bands of 7.1, 3.05, and 2.7 kb. Thus, HIV-producing 251-MG sublines contained only approximately 1 or 2 copies of viral DNA per cell, none of which was in the form of unintegrated HIV DNA. It is unclear from these data whether the HIV DNA in these glial cell lines is integrated into the cellular DNA or whether it is present as an extrachromosomal element.

Steady-state levels of selected cellular gene transcripts in HIV-positive glial cells. To investigate possible changes in cellular gene expression in HIV-positive 251-MG sublines, total cellular RNA was analyzed by Northern blot analysis by using several probes for "housekeeping" genes such as β-actin and β-tubulin as well as "luxury" functions such as class I and class II histocompatibility antigens (HLA-B7, HLA-DR-β) and GFAP. Steady-state mRNA levels of all of these cellular genes were unchanged in HIV-positive lines as compared with the G418-resistant, HIV-negative control 251-MG/neo (data not shown), in contrast to the findings of Kannagi and colleagues in HIV-positive and HIV-negative cells (24).

DISCUSSION

Comparison of HIV life cycle in glial cells directly infected with intact HIV and in HIV-positive lines established after transfection. These studies confirm reports from various laboratories that human brain-derived cell lines are susceptible to a low-level infection by HIV (7, 8, 11). We now provide unequivocal proof that GFAP-positive astrocytes can be infected with HIV in vitro and that HIV can persist in these cells for prolonged periods of time. As has been noted previously (7, 8, 11), the infection of glioma-derived cell lines with intact HIV is characterized by minimal virus replication, inability to detect viral antigens by immunofluorescence staining, and lack of virally induced cytopathic effects. In contrast, HIV-infected astrocytes in the brain express detectable levels of both viral antigens (40) and virus particles (14). We now demonstrate such high-level infection of glial cells by HIV in vitro.

The characteristics of HIV infection in U-251MG cells directly infected with intact HIV and highly HIV-positive 251-MG sublines derived by HIV DNA transfection are compared in Table 3. In both situations, HIV persisted in the cells for prolonged periods (several months) and did not induce syncytium formation or cytolysis. However, only in

TABLE 2. Growth kinetics of G418-resistant U-251MG sublines[a]

Time point (days)	Protein determination (mg)			[^{3}H]thymidine uptake (cpm)		
	251-MG/E_a	251-MG/E_b	251-MG/Neo	251-MG/E_a	251-MG/E_b	251-MG/Neo
0	0.044 (0.004)	0.056 (0.017)	0.057 (0.002)	518 (219)	499 (254)	781 (278)
1	0.059 (0.010)	0.073 (0.009)	0.115 (0.011)	2,226 (575)	5,076 (1,172)	4,080 (371)
3	0.093 (0.012)	0.175 (0.025)	0.147 (0.011)	4,499 (1,840)	6,199 (1,670)	5,835 (1,927)
5	0.132 (0.006)	0.238 (0.010)	0.282 (0.026)	4,777 (751)	3,651 (789)	3,163 (1,003)
7	0.277 (0.016)	0.242 (0.016)	0.329 (0.059)	1,323 (225)	1,153 (312)	1,131 (185)

[a] Results are expressed as means of triplicate repeat experiments, with standard deviations given in parentheses.

the case of HIV-positive 251-MG sublines established after transfection with infectious molecular clones of HIV could viral antigens be detected in cells by immunofluorescence staining, and only in supernatants from cultures of these cells was HIV present at high levels. Our data show that there is a significant difference in the level of HIV RNA and DNA in directly infected U-251MG cells as compared with sublines established via transfection (Table 3). Possible explanations for this observation include the following.

(i) Some defect in HIV replication or expression may exist in these cells. It is interesting to note that unintegrated HIV DNA cannot be detected in glial cells or in the 251-MG sublines, suggesting a possible defect in reverse transcription or integration.

(ii) Rapid downregulation (23) of scarce (12) HIV/CD4 receptor (25) molecules on these cells may prevent secondary infection of cells with progeny virus produced by HIV-infected U-251MG cultures.

(iii) Certain HIV strains show a preferential neurotropism (7, 26), and it is possible that the HIV strain used for direct infection of glial cells may have been defective in some manner. However, studies with other virus isolates (including the SF_2 isolate of HIV as well as a variety of virus clones maintained in our laboratory) produced results very similar to those presented here (our unpublished observations), making this unlikely.

HIV load per cell correlates with observed changes in cellular morphology. HIV infection of cultured glioma cells occurred in the absence of the cytopathic effects (cytolysis, syncytium formation) typical of HIV-infected human T lymphocytes (25). Nonetheless, the morphology of HIV-positive sublines established after transfection with HIV DNA was markedly different from that of HIV-negative controls. Intriguingly the line (251-MG/E_a) which contained the greatest copy number of HIV RNAs per cell also exhibited the slowest growth and most significant morphological abnormalities.

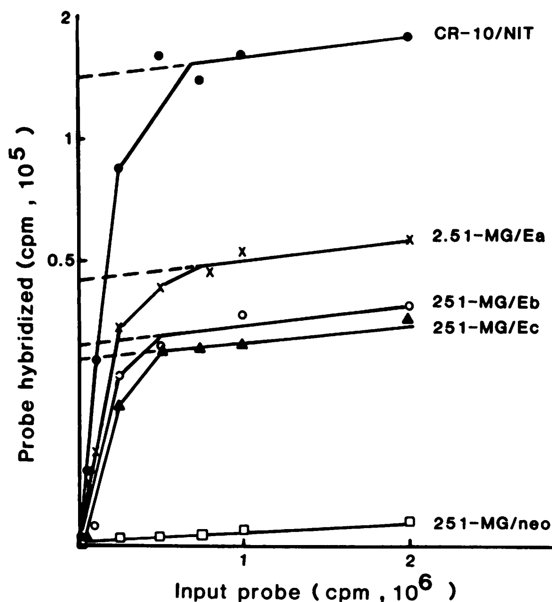

FIG. 5. Hybridization saturation curves for HIV RNA load in HIV-positive 251-MG sublines. Cells were washed in phosphate-buffered saline, dissolved at a density of 5×10^6 cells per ml in 4 M guanidium isothiocyanate (or 10^7 cells per ml in the case of CR10/N1T cells), and hybridization saturation curves were calculated as described in Materials and Methods. The HIV RNA load per cell was determined on the basis of the intersection of extrapolated saturation curves with the ordinate.

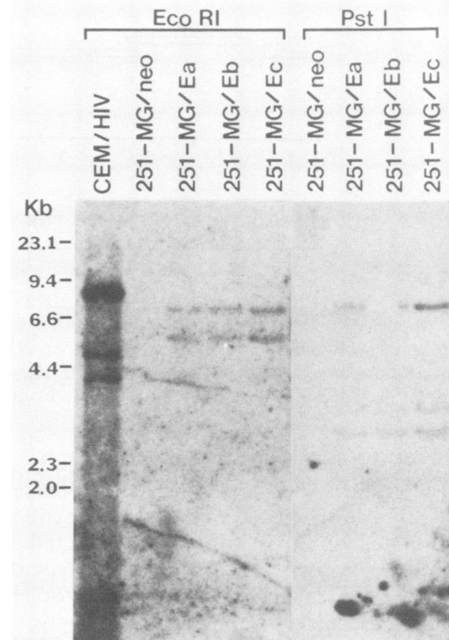

FIG. 6. Identification of HIV DNA in G418-resistant U-251MG sublines by Southern blot hybridization. Genomic DNA (10 μg) from the indicated cell lines was analyzed in each lane after digestion with the indicated restriction enzymes. The HIV DNA probe used was a nearly full-length, electroeluted, radiolabeled fragment (described in Materials and Methods). Size markers were ^{32}P-end-labeled *Hin*dIII digests of λ DNA.

Lack of cytolysis of glial cells despite highly productive, persistent infection with HIV. Previous cotransfection experiments with *neo* and HIV DNA in human rhabdomyosarcoma cells (1) or mouse fibroblasts (27) failed to result in establishment of chronic HIV-producing cell lines. Although the explanation for this is unclear, it is interesting to note that the highly HIV-positive, neo^+ U-251MG sublines described in the present work do not undergo virally induced cytolysis. This may result, in part, from the low-level expression of the CD4 molecule (12), since this molecule appears to be the cellular receptor for HIV (25) and also to be intimately involved in virally induced cell fusion and lysis (34). Observations in monocyte cultures (19) and in a monocytoid cell line and its subclones (2) support the theory that low-level T4 expression correlates with a noncytopathic infection. It is unlikely that this lack of cytolysis is due to any lack of cell surface expression of HIV envelope glycoproteins, since the 251-MG cell lines described here are

TABLE 3. Characteristics of infected versus transfected U-251MG cells

Property	Transfection	Direct infection
Cytopathic effects	No	No
Morphological abnormalities	Yes	No
HIV antigens	>50%	No
RT activity	$>100 \times 10^3$	$<1 \times 10^3$
No. of HIV RNA copies/cell	>1,000	<10

efficient virus producers and must therefore express considerable quantities of both gp120 and gp41.

Implications. Our data suggest that HIV may productively infect astrocytes in the brain with concomitant changes in the phenotype of such cells, including alterations in the expression of as yet unknown genes (as has been described in HIV-infected T-cell lines [6, 24]). It is possible that this may contribute to the pathogenesis of the AIDS dementia complex through effects on surrounding uninfected cells.

It is probable that glial cells in the brain may express higher levels of the CD4 receptor than the glioma cell lines used in our studies (18). Immature brain cells may express especially high levels of this molecule, since (i) fetal neural cells are highly permissive to HIV infection (39), (ii) pediatric AIDS patients are especially vulnerable to neurological damage (3), and (iii) cell surface expression of receptors for other viruses is thought to be controlled by the state of cellular differentiation (41). Thus, HIV infection of astrocytes in the brain (particularly in children) may be more akin to the persistent, replicative infection which we observe in cell lines established after transfection and selection rather than to the direct infection of glial cells with intact HIV in vitro.

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