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Persistent Human Immunodeficiency Virus Type 1 Infection in Human Fetal Glial Cells Reactivated by T-Cell Factor(s) or by the Cytokines Tumor Necrosis Factor Alpha and Interleukin-1 Beta

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Human immunodeficiency virus type 1 (HIV-1) infection of the brain has been associated with a severe dementing illness in children and adults. However, HIV-1 antigens are most frequently found in macrophages and microglial cells. To determine the extent of susceptibility of neuroglial cells to infection, the HIV-1 genome was introduced into cells cultured from human fetal brain tissue. Astroglial cells rapidly transcribed the viral genome producing high levels of p24 protein and infectious virions which peaked two to three days posttransfection. Thereafter HIV-1 genome expression progressively diminished and a persistent phase of infection developed during which neither virus nor viral proteins could be demonstrated by immunodetection methods. Cocultivation with CD4$^+$ T cells at any time during the persistent infection resulted in resumption of p24 synthesis and virus multiplication. The release of persistence did not require direct cell-cell contact between the glial and T cells, since separation of the two cell types across a permeable membrane resulted in a delayed but similar resumption of p24 synthesis and virus multiplication. The persistently infected glial cells could also be stimulated to produce viral p24 protein if either tumor necrosis factor alpha or interleukin-1 beta was added to the medium without T cells present. These results suggest that astrocytes may serve as an undetected reservoir for HIV-1 and disseminate the virus to other susceptible cells in the brain upon triggering by some cellular or biochemical signal.

Human immunodeficiency virus type 1 (HIV-1) infection of the human brain has been associated with neurological dysfunctions in both adults and children. Such neurological complications in AIDS have been described as an HIV-1 encephalopathy and commonly characterized as a progressive dementia in adult AIDS and loss of developmental milestones in pediatric AIDS (7, 8, 30). The histopathology of HIV-1 in the brain has identified some neuronal cell loss and myelin pallor, suggesting viral damage to these cells. However, viral antigen is found chiefly in microglial cells, the macrophage of the brain, and in multinucleated giant cells. There have only been a few instances of viral antigen found in neurally derived cells including astroglial and oligodendroglial cells and neurons (15, 36). To explain the disparity between the neurological deficits and lack of evidence of HIV-1 in neural cells, mechanisms that involve indirect effects of HIV-1, such as release of toxic substances from infected leukocytes that cause neural cell injury, have been proposed (13, 34). Tissue culture experiments, however, have shown that HIV-1 can directly infect human cell lines derived from glial or neuronal tumors (2, 3, 5, 6, 16, 19, 20) even in cells that do not express the virus receptor CD4 (5, 16, 20). To investigate the consequence of HIV-1 infection in normal human glial cells, we have established a tissue culture system with human fetal brain cultures comprising astroglial and oligodendroglial precursor cells and have introduced HIV-1 into these cells by infection with virions or transfection with a molecular proviral clone. We present evidence here that offers an additional explanation for the observations in AIDS encephalopathy. The data show that HIV-1 can establish a long-term persistence in glial cells during which neither viral antigen nor virions can be detected. However, in the presence of CD4$^+$ T cells, whether in direct cell-cell contact or separated by a permeable membrane, persistently infected glial cells resume synthesis of p24 protein and produce infectious virus. Several cytokines that are suspected of causing neural cell damage, tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), can also induce viral protein synthesis from the persistently infected glial cells. It is possible that the inability to detect HIV-1 in neurally derived cells in vivo is due to the observed persistence that is demonstrated in vitro. The indirect effect of HIV-1-infected macrophages or T cells may be in part due to their ability to stimulate the persistently infected glial cells to once again produce virus. Cytokines that regulate HIV-1 expression have been described for cells of macrophage and monocyte lineages (9, 12). We suggest that similar cytokines play an equally important role in the pathogenesis of HIV-1 in the human nervous system. These observations may be more applicable to vertically transmitted HIV-1 infection during fetal development and need to be tested in HIV-1-infected adult and pediatric brain tissue.

MATERIALS AND METHODS

Cell cultures from brain. Cultures from human fetal brain tissue were prepared as previously described (23) with minor modifications. Brain tissue was dissected from 9- to 14-week human fetuses, mechanically disrupted by aspiration through a 19-gauge needle, washed in Eagle minimum essential medium and plated into poly-D-lysine (0.1 mg/ml in distilled water)-treated tissue culture flasks. Each brain specimen was plated separately without pooling of tissues from similar or different gestational ages. Cultures were

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maintained and fed every 3 to 4 days with Eagle minimum essential medium plus 10% fetal bovine serum. To prepare pure cultures of astrocyte cells, the cultures were first refed with serum-free medium and placed in an orbital shaker (210 rpm) at 37°C for 16 h. Cells released from the cultures were discarded, while the adherent cells were refed with medium with serum. For serial passage, cells were harvested by using 0.025% trypsin and 0.005% EDTA, counted in a hemocytometer, and plated at 10^5 cells per cm^2.

Transfection procedure. The calcium phosphate precipitation technique was used for transfection. Uncleaved plasmid DNA (5 μg) was used for each assay. The DNA was precipitated in HEPES buffer, pH 7.1 (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]), with a 125 mM $CaCl_2$ final concentration. The DNA in HEPES buffer was kept at room temperature for 30 min to allow the precipitate to form. The cell cultures were washed in HEPES buffer prior to adding the DNA. Following a 4-h incubation, the cells were treated with 15% glycerol, washed, and incubated at 37°C until harvest.

Immunodetection assays. The cells were plated on glass coverslips, fixed in acetone and methanol for 30 min at −20°C, washed in phosphate-buffered saline (PBS; pH 7.1), and blocked with 3% normal goat serum. The cells were then incubated in the primary mouse antibody (anti-glial fibrillary acidic protein [Chemicon], anti-gp120/160 [Olympus], anti-p24 [Olympus], or anti-gp41 [Genetic Systems]) in blocking buffer for 90 min at room temperature. The cells were then washed in PBS and incubated with either alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim) at a dilution of 1:50 or biotin-conjugated horse anti-mouse immunoglobulin G and then with horseradish peroxidase complex. Color was developed with chromogens 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride for alkaline phosphatase or diaminobenzidine for peroxidase label. All incubations with antisera were conducted at 4°C.

Cell culture separations. Transwell chambers are porous-bottom culture dishes which fit into divided wells of cluster plates (Costar), forming a two-compartment system. The two compartments are separated by a 0.4-μm-pore-size polycarbonate membrane which serves as the bottom of the Transwell chamber and allows for rapid diffusion between compartments without cell-cell contact.

Cytokines. All cytokines used were either human recombinant or derived from a human cell source (Boehringer Mannheim). Cytokines were stored in aliquots at −20°C following reconstitution in minimum essential medium with 10% fetal calf serum. Aliquots were thawed only once. Concentrations of cytokines were as follows: TNF-α, 10 ng/ml; TNF-β, 5 ng/ml; granulocyte macrophage–colony-stimulating factor (GM-CSF), 400 U/ml; fibroblast growth factor beta, 40 ng/ml; gamma interferon, 500 U/ml; IL-1β, 10 U/ml; IL-2, 50 U/ml; and IL-6, 500 U/ml.

RESULTS

HIV-1 introduction into fetal brain cells. Independent cultures of human fetal neuroglial cells (HFGC) were established from brain tissue derived from 9- to 14-week human fetuses (23). The HIV-1 genome was introduced into these cultures either through infection with virion NL4-3 or through transfection with the infectious molecular clone pNL4-3 (1). Levels of p24 antigen and reverse transcriptase were measured from supernatant fluids collected at different times following cell culture treatment. As expected, since these cultures are $CD4^-$, virus infection resulted in very small amounts of newly synthesized p24 antigen and reverse transcriptase (data not shown). Progeny virus could only be detected after prolonged coculture with A3.01 cells, a human T-cell line (10), similar to previous reports of the neurotropism of HIV-1 in human fetal brain tissue in which cocultivation was also necessary for detection of progeny virus (2). To determine whether cell-cell contact could lead to infection of HFGC, NL4-3-infected A3.01 T cells were cocultivated with uninfected human fetal astrocytes. Twenty-four hours later, the A3.01 cells were lysed with an anti-CD4 antibody and complement. Seventy-two hours following lysis of the A3.01 cells, approximately 0.5% of the astrocytes expressed gp41, as determined by immunoperoxidase staining (Fig. 1). Antigen was expressed throughout the processes of the astrocytes, suggesting that infection of the astrocyte had occurred. Transfection of HFGC with pNL4-3 DNA resulted in a rapid synthesis of p24 protein within hours after transfection, as indicated in Fig. 2A. Reverse transcriptase measurements paralleled the p24 protein levels but generally were less sensitive at low protein concentrations (data not shown). Daily measurements of p24 in the culture fluids over several weeks generated the growth profile of HIV-1 in the glial cells which is shown in Fig. 2B. After reaching peak levels of viral protein during the first few days, p24 levels began to decline. From approximately 15 days after transfection until sampling was discontinued at 30 days, p24 protein was not detectable above background levels.

Infectious progeny HIV virions were produced during the time of p24 increase, as demonstrated by infection of A3.01 cells cocultivated with transfected glial cells. Viral synthesis in the A3.01 cells was 4 orders of magnitude greater than that seen in human fetal glial cells (see Fig. 4, right-hand column). This observation is consistent with other reports of the susceptibility of human glial cells to HIV-1 infection (2, 4). However, regardless of the stage of HIV-1 infection in the glial cultures, no cytopathic effects were observed, nor was there any indication of morphological alterations or syncytial cell formation. However, A3.01 cells that became infected following cocultivation with the transfected glial cells demonstrated both syncytia and multinucleated giant-cell formation.

Identification of HIV-1 proteins in glial cells. Identification of HIV-1 proteins in the astrocyte population of cells was made by using immunocytochemistry. Monoclonal antibodies to p24 and gp120/160 recognized HIV-1 antigens in HFGC 48 h after transfection, as demonstrated in Fig. 3A and 3B. Both antigens were present in the cytoplasm of the cells which were morphologically similar to astrocytes, with p24 being most prominent in the perinuclear region, though some staining of the nucleus with antibody to p24 was also noticed (Fig. 3B). Nearly 10% of the astrocytes expressed HIV-1 antigens. The physical presence of HIV-1 structural proteins in these cells was further confirmed by immunoblot analysis of cell extracts (Fig. 3C). Lane 1 shows the presence of the envelope glycoprotein precursor gp160 as a minor band, most of which has been cleaved to form gp120. Lane 2 shows the presence of the core protein p24. Attempts to detect HIV-1 antigens in HFGC at 14 days or later after transfection by either immunostaining or immunoblotting were unsuccessful. Also, enzyme-linked immunosorbent assays (ELISAs) of cell culture fluids at these later times did not demonstrate p24 protein above background levels of the assay. Confirmatory identification of HIV-1 proteins in astrocytes was made by double labelling of transfected cells

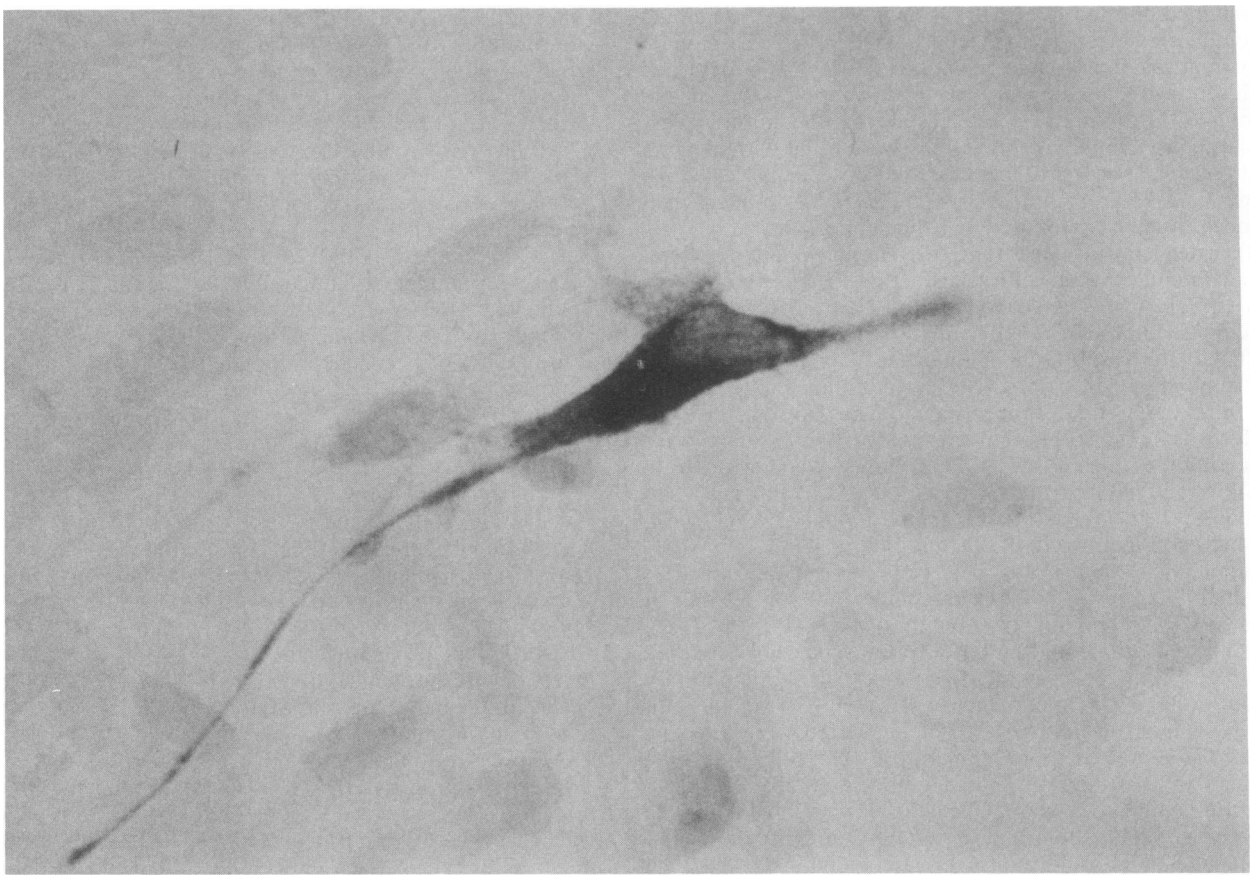


FIG. 1. Infection of human fetal astrocytes with HIV-1 by cell-cell contact with HIV-1-infected A3.01 T cells. A3.01 cells (10^4 cells) were infected with NL4-3 and then cocultivated with 10^6 uninfected human fetal astrocytes. Twenty-four hours after cocultivation, the cultures were incubated with an anti-CD4 immunoglobulin G (5 μg/ml) and rabbit complement (undiluted, 35 μl/ml of culture supernatant) to lyse the T cells. Seventy-two hours after lysis of the A3.01 cells, approximately 0.5% of the astrocytes expressed gp41 as demonstrated with an anti-gp41 mouse monoclonal antibody and immunoperoxidase method.

with monoclonal antibodies to viral gp120/160 and the astrocyte-specific intermediate filament glial fibrillary acidic protein (GFAP). Staining of infected cells showed that GFAP-positive cells also reacted with antibody to gp120/160.

Establishment of HIV-1 persistence in glial cells. To test whether low levels of virus that could go undetected by these assays were present in the cultures 2 weeks after transfection, an experiment, graphically depicted in Fig. 4, was done. Duplicate cultures that were 95% astrocytes were transfected with pNL4-3. Supernatant fluids from one set of cultures were collected on days indicated (2, 6, 14, 21, 29, 35, and 42 days) and used to treat A3.01 cells. The other set of astrocyte cultures were directly cocultured with A3.01 cells at similar times after transfection. As an indication of the presence of HIV-1, p24 protein was measured in the A3.01 cultures 12 days after either cocultivation or treatment with supernatants. Regardless of whether cocultivation was initiated at early or late times after transfection, A3.01 cells became infected and produced high levels of p24. However, only supernatant fluids taken from the transfected astrocytes prior to day 14 could initiate infection in the target A3.01 cells. Also, levels of p24 produced in the target lymphocytes that were treated with day 6 and day 14 supernatant fluids were 10-fold lower than the level reached with the supernatant from day 2, indicating a gradual reduction in the amount of virus produced in the astrocyte cells with time. This experiment was reproduced with phytohemagglutinin-stimulated human peripheral lymphocytes as target cells. These results indicate that HIV-1-infected human fetal glial cells initially undergo a noncytopathic productive infection that becomes a nonproductive or persistent infection. During this time of persistence, virus could be released from the HFGC cultures by cocultivation with $CD4^+$ T lymphocytes.

Reactivation of HIV-1 from persistently infected glial cells. Since the T lymphocytes attached to the astrocyte cells during cocultivation could not be dislodged, it was not clear whether virus was transferred through cell-cell contact or new virus synthesis was initiated in the astrocyte cells upon some signal from the T cells. To distinguish between these alternatives, an experiment similar to that described above for Fig. 4 was repeated but also included persistently infected astrocyte cells that were either cocultivated directly with A3.01 cells or separated from the A3.01 cells by using a Transwell chamber. Figure 5 shows the results of this experimental design. Fifteen days after transfection, when neither p24 protein nor virus could be demonstrated in the supernatant fluids, A3.01 cells were either placed directly in wells with the astrocytes or placed in the Transwell chamber above the astrocyte cells. Within 24 h, p24 protein was

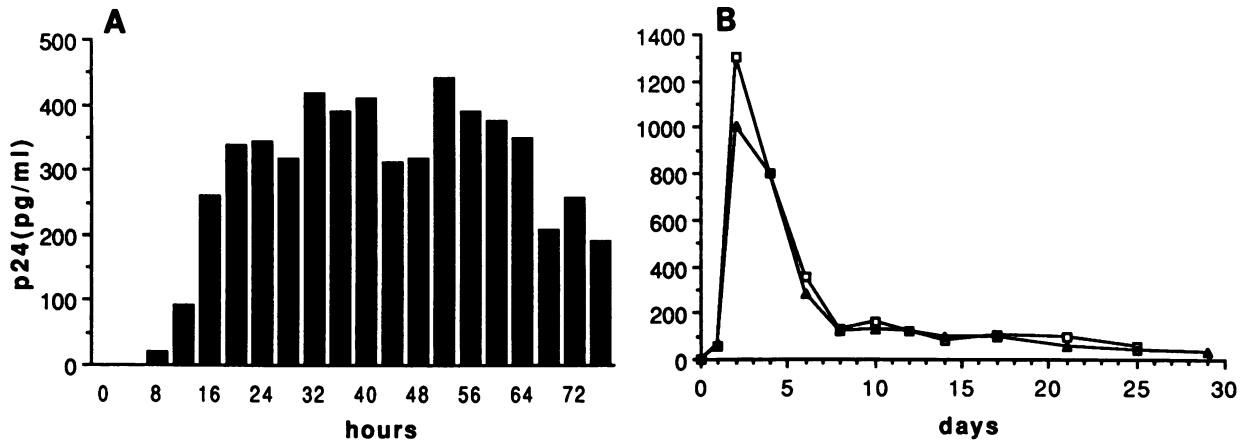


FIG. 2. Transfection of human fetal glial cell cultures with pNL4-3 DNA. (A) HFGC were transfected as described in the text. p24 protein concentration in the supernatant fluid was determined every 4 h for 3 days by using an antigen capture ELISA (Coulter). At each time interval, the culture medium was collected and replaced with fresh medium. Samples of 500-μl were analyzed by ELISA (Coulter) for HIV-1 p24. For quantitative estimation of p24 antigen, a standard curve was made from twofold dilutions of a predetermined amount of p24. (B) Two cultures of human fetal glial cells were established from separate brain tissues. One culture (□) represented a heterogeneous population of glial cells (astrocytes and precursor cells of oligodendrocytes and neurons), while the other culture (△) was enriched for astrocytes. The cultures were transfected with pNL4-3 and p24 protein, measured as described above, with a complete medium change at each sample collection. Each datum point represents a mean value of three experiments. Five independent experiments with cells from different gestational ages, 9 to 14 weeks, have shown similar results. The *y* axis (picograms of p24 per milliliter) is the same for both graphs.

detectable in the cocultured wells and continued to increase to very high levels, similar to the results shown in Fig. 4. Both p24 protein and gp120 protein were identified in the A3.01 cells by immunostaining. Detection of p24 synthesis in the Transwell chamber supernatant fluids required an additional 4 days, but eventually increased to high levels. In order for the A3.01 cells to become infected, HIV-1 had to be released from the persistently infected astrocytes and infect the T lymphocytes through the shared medium, which took several days longer than direct cocultivation (Fig. 5). This result implicates a cellular signal from the target T cell which could stimulate virus multiplication from the persistently infected astrocytes.

Cytokine effect on release of persistence in glial cells. Several cytokines, such as TNF-α, IL-1 and -6, and GM-

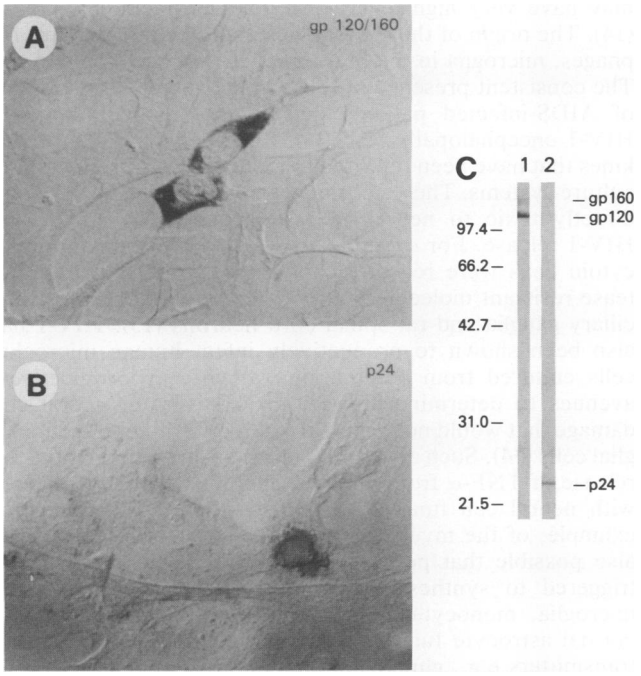
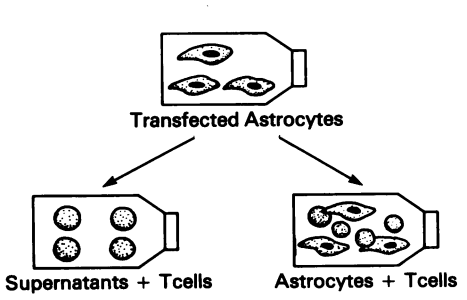


FIG. 3. Immunological detection of HIV-1 antigens: human fetal astrocytes expressing gp120/160 (A) and p24 proteins (B) 48 h after transfection. Nomarski optics were used; magnification, ×200. (C) Immunoblotting of proteins from cell extracts of HFGC prepared with 1% Nonidet P-40 and electrophoresed through a sodium dodecyl sulfate–10% polyacrylamide gel. Proteins were electroblotted to nitrocellulose filters and detected with antibodies to gp120 (lane 1) and p24 (lane 2) as described in Materials and Methods.



Days post transfection	p24 protein concentration (ngr/ml) measured 12 days after A3.01 cell inoculation	
2	80.0	140.0
6	9.8	50.5
14	<0.01	87.6
21	<0.01	110.0
29	<0.01	100.6
35	<0.01	120.2
42	<0.01	100.0

FIG. 4. p24 protein concentrations in A3.01 cells either treated with supernatant fluids from transfected fetal astrocytes (Supernatants + Tcells) or cocultivated directly with transfected astrocytes (Astrocytes + Tcells). Inoculum for the A3.01 cells was taken at times after transfection as indicated on the chart.

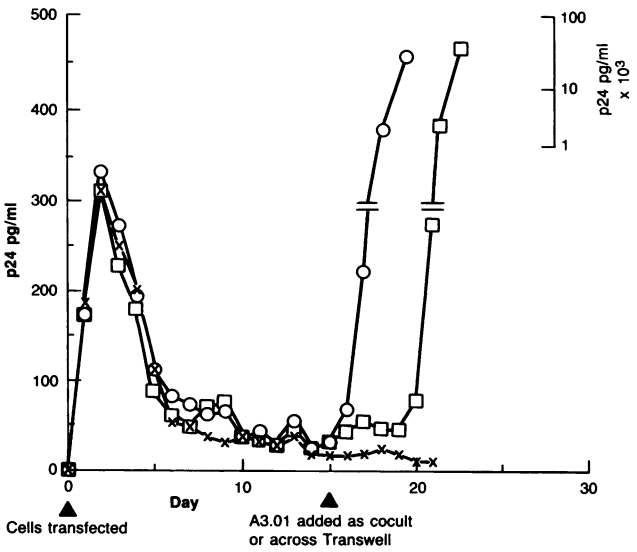


FIG. 5. Reactivation of HIV-1 in persistently infected cells in the presence of A3.01 cells. HFGC were transfected with pNL4-3 DNA and allowed to reach a state of viral persistence. On day 15, A3.01 cells were added to the cultures either by direct cocultivation (○) or by separation on a Transwell membrane (□). A control culture of transfected HFGC was not exposed to A3.01 cells (×). All datum points represent the mean value from three experiments. Ordinate in the upper right of graph represents log values applied to samples above parallel (=) lines.

CSF, have been shown to stimulate HIV-1 multiplication from human monocyte cell lines (11, 25–27). We tested the possibility that soluble cytokines added to the medium of persistently infected glial cells produce effects similar to those demonstrated by the addition of A3.01 cells in the Transwell. The results in Fig. 6 show that TNF-α and IL-1β were able to induce HIV-1 production from the persistently infected glial cells but that GM-CSF, IL-2 and -6, and fibroblast growth factor beta, TNF-β, and gamma interferon could not. Supernatant from TNF-α- and IL-1β-treated glial cells was infectious, as demonstrated by incubation of the supernatant with A3.01 cells (data not shown). Although the mechanism for HIV-1 induction in this experiment is unknown, the results demonstrate that certain cytokines, independent of the presence of T cells, are able to shift glial cells from a persistent to a productive infection.

DISCUSSION

The HIV-1 genome can be rapidly expressed in human fetal glial cells upon transfection of an infectious molecular clone. The result of HIV-1 expression in glial cells is an initial productive infection, as evidenced by high levels of p24 protein production (Fig. 2A and B) and virus multiplication (Fig. 4). However the productive phase of infection diminishes after 3 to 5 days. HIV-1 protein expression in glial cells appears to occur only in this time of productive infection, since at later times neither viral proteins nor infectious virus is demonstrable (Fig. 4). The HIV-1 genome, however, seems to remain in a nonproductive or persistent state that can be triggered to resume viral synthesis either by a factor(s) from T lymphocytes, as demonstrated by the experiment in Fig. 5, or by cytokines TNF-α and IL-1β, as shown in Fig. 6.

These observations offer a new insight and suggest an important possible mechanism which explains HIV-1-associated encephalopathy, e.g., the presence of HIV-1 in glial cells may go undetected during a persistent infection but, upon activation by an immune cell factor(s) such as cytokines, may cause neural cell dysfunction or damage. The development of HIV-1 persistence in normal human fetal glial cells is supported by observations made by using human glioma and neuron-derived cell lines in culture (3, 5, 6, 16, 19, 20). Infection in some of these cell lines involved a CD4-independent pathway and, like the fetal glial cells, did not result in a cytopathic infection. Several cytokines, including TNF-α, IL-6, and GM-CSF, have been reported to induce HIV-1 replication from persistently infected human monocyte cell lines (11, 25–27). Human astrocytes themselves produce TNF-α and IL-1 and -2 as well as respond to phorbol esters and interferons which can modulate cellular activities (21, 28). For example, phorbol myristic acid has been able to augment virus multiplication by stimulation of protein kinase C (18). In HFGC cultures, however, addition of phorbol myristic acid did not augment the release of p24 into the supernatant fluids compared with that by non-phorbol myristic acid-treated cells during either the productive or the persistent phase of infection (data not shown). IL-1β, on the other hand, upregulates the HIV-1 long terminal repeat in primary rat astrocytes expressing chloramphenicol acetyltransferase activity (31) and is able to reinitiate virus production from the persistent infection described here. Neither IL-6 nor GM-CSF could produce the same result in HFGC (Fig. 6).

Cerebrospinal fluid in patients with HIV-1 encephalopathy may have very high levels of cytokines, including TNF-α (14). The origin of these cytokines could be infected macrophages, microglia in the brain, monocytes, or lymphocytes. The consistent presence of these infected cells in the brains of AIDS-infected patients defines the histopathology of HIV-1 encephalopathy (17, 33). Such cells can elicit cytokines that have been reported to induce HIV-1 production in culture systems. These cells may also release factors that are directly toxic to neural cells independent of stimulating HIV-1 release. For example, HIV-1-infected human monocytoid cells were recently shown to secrete a small, protease-resistant molecule that was toxic to embryonic chick ciliary ganglia and rat spinal cord neurons (13). HIV-1 has also been shown to productively infect human microglial cells cultured from adult brain, which may provide new avenues to determine indirect mechanisms of neural cell damage that would not necessarily involve direct infection of glial cells (34). Such an indirect mechanism could involve the release of TNF-α from infected microglia, which interferes with neural cell function or causes direct cell death, for example, of the myelinating oligodendrocyte (17, 35). It is also possible that persistently infected astrocytes may be triggered to synthesize HIV-1 proteins by factors from microglia, monocytes, or lymphocytes. Interference with normal astrocyte functions such as metabolisms of neurotransmitters e.g., glutamate, antigen presentation to T cells by major histocompatibility complex restriction, and blood-brain barrier integrity in HIV-1 persistently infected cells could become part of the pathogenesis of HIV-1 infection in the nervous system, as demonstrated by HIV-1 protease cleavage of the astrocyte-specific intermediate filament, GFAP (32). To better understand the interactions of HIV-1 and glial cells, we are currently defining the transcriptional

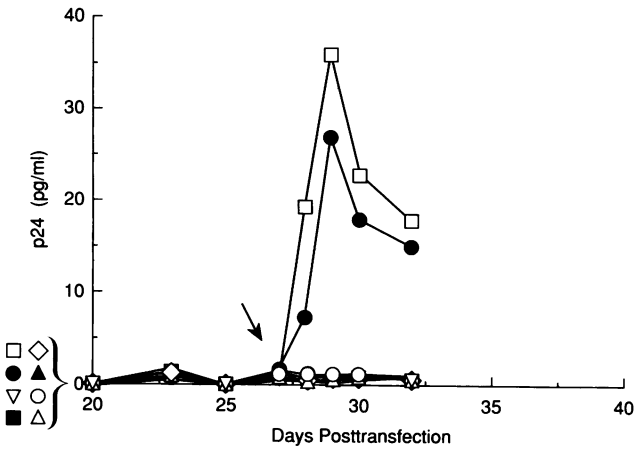


FIG. 6. Reactivation of HIV-1 in persistently infected cells after administration of cytokines. At day 27 (↘) posttransfection with pNL4-3, cytokines were added to the medium and replaced for 2 consecutive days. Concentrations of cytokines are described in Materials and Methods. □, TNF-α; ●, IL-1β; ▽, IL-2; ■, GM-CSF; ▲, gamma interferon; △, fetal growth factor beta; ○, IL-6; ◇, TNF-β.

control of the productive and persistent course of HIV-1 in human fetal glial cells (29) and testing other known cytokines for their abilities to release persistence from astrocyte cultures without the presence of human lymphocytes. We are also examining autopsy tissue sections from brain samples from pediatric patients with AIDS to localize mRNA for cytokines in regions of HIV-1-induced histopathology. The recent observations of HIV-1 in human fetal brain tissue (22, 24) and the description of a shared immunogenic epitope of HIV-1 gp41 and astrocyte cells (37) emphasize the need for a model for HIV-1 infectivity in normal human glial cells to study viral pathogenesis of central nervous system involvement in pediatric as well as adult AIDS.

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