Human Immunodeficiency Virus 1 Envelope Proteins Induce Interleukin 1, Tumor Necrosis Factor α, and Nitric Oxide in Glial Cultures Derived from Fetal, Neonatal, and Adult Human Brain

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Summary

Although microglia are the only cells found to be productively infected in the central nervous system of acquired immunodeficiency disease syndrome (AIDS) patients, there is extensive white and gray matter disease nonetheless. This neuropathogenesis is believed to be due to indirect mechanisms other than infection with human immunodeficiency virus 1 (HIV-1). Cytokines and toxic small molecules have been implicated in the clinical and histopathological findings in CNS AIDS. Previously, we have demonstrated in rodent glial cultures the presence of biologically active epitopes of gp120 and gp41 that are capable of inducing interleukin 1 and tumor necrosis factor α. In this study, we map the HIV-1 envelope epitopes that induce nitric oxide, inducible nitric oxide synthase, interleukin 1, and tumor necrosis factor α in human glial cultures. Epitopes in the carboxy terminus of gp120 and the amino terminus of gp41 induce these proinflammatory entities. In addition, we compare HIV-1 infection and pathology in glial cells derived from human brain taken at different states of maturation (fetal, neonatal, and adult brain) in an effort to address some of the clinical and histological differences seen in vivo. This study demonstrates that, in the absence of virus infection and even in the absence of distinct viral tropism, human glia respond like rodent glia to non-CD4-binding epitopes of gp120/gp41 with cytokine and nitric oxide production. Differences among fetal, neonatal, and adult gial cells' infectivity and cytokine production indicate that, in addition to functional differences of glia at different stages of development, cofactors in vitro and in vivo may also be critical in facilitating the biological responses of these cells to HIV-1.

HIV-1 disease produces a primary neurologic/neuropsychiatric disorder, although the precise contribution of virus to either brain or spinal cord pathology or to clinical symptoms is thought not to be due to actual infection of neurons or oligodendrocytes (1). Infection in fetal brain is difficult to detect even by PCR or in situ hybridization, and virus isolation from these brains is rare. When infection occurs in fetal brain, it does so in few cells and may be latent or defective (2). HIV-1 nucleic acids and proteins are more easily detected in pediatric than in fetal brain; nevertheless, infection in neonatal brain is at a lower level than in adult brain, and productive infection is not usually evident (3).

Because pathology occurs in cells not infected with virus, it is possible that cytokines and small toxic molecules are indirectly at work in central nervous system (CNS)1 AIDS (4). In AIDS patients, HIV-1 infection increases the levels of several proinflammatory cytokines, such as IL-1, IL-6, and TNF-α in vivo and in vitro (for review see reference 4). TNF-α and IL-1 have been found to be elevated in CNS AIDS brain tissue, with significantly higher amounts in demented HIV-1–infected patients (5). Levels of TNF-α have been correlated with spinal cord vacuolar

1Abbreviations used in this paper: ANOVA, analysis of variance; AZT, azidothymidine; CNS, central nervous system; CYTO, cytokines; GCT, giant cell tumor supernatant; iNOS, inducible nitric oxide synthase; Dil-α-LDL, acetylated low-density lipoprotein; mRNA, messenger RNA; NO, nitric oxide; NO−, total nitric oxide; RCA-1, Ricinus communis agglutinin 1; WR, wild type.
myelopathy and brain encephalitis (5). The findings of HIV-1 induction of IL-1, TNF-α, and IL-6 in macrophages and glia are interesting since all three of these cytokines up-regulate HIV-1 replication in T cells or MO (1, 6). The primary cell type infected in vivo in CNS AIDS is the microglial cell (1). The CD4-independent infections in human astrocytes (malignant or primary fetal) were usually transient, nonproductive/latent, or partially defective and did not lead to in vitro cytopathicity (7–9). In human adult brain, CD4+ macrophages were shown to fuse and die after in vitro infection by a macrophage-tropic strain of HIV-1 (10), but there is some controversy remaining as to whether human microglia are CD4+ and whether they can be infected (11, 12).

Recently, human macrophages, astrocytes, and astrocytomas have been shown to produce nitric oxide (NO) via inducible NO synthase (iNOS) in response to cytokines and gp120 (13–16). iNOS has been seen in AIDS retina, suggesting NO as a possible mechanism for CNS damage (17); NO damages neurons and oligodendrocytes in vitro. Because gp120 and gp41 are able to induce IL-1 and TNF-α in glial cultures (18, 19), we have mapped epitopes in HIV-1 envelope that induce IL-1, TNF-α, and iNOS at the protein and messenger RNA (mRNA) level. In the absence of significant productive infection, gp120 carboxy terminus and gp41 amino terminus epitopes give rise to cytokines and NO production in human glial cultures from tissue taken at different stages of development.

Materials and Methods

Primary Human Glial Cells: Establishment of Cultures and Glial Cell Type Identification. The human glial cultures were established from adult, neonatal, and fetal brain tissue. The adult and neonatal brain tissue was obtained from epilepsy patients undergoing surgical resections for intractable seizures. Tissues from four adults were obtained; the patients’ average age was 23.8 ± 4.0 yr (range 18.0–32.0 yr). The tissue was derived from frontal and temporal lobe regions. The neonatal brain tissues were procured during surgical resections for intractable seizures. Tissues from four adults were obtained; the patients’ average age was 23.8 ± 4.0 yr (range 18.0–32.0 yr). The tissue was derived from frontal and temporal lobes. The neonatal brain tissues were procured from 10 cases, with an average age of 3.1 ± 2.0 yr (range 1.4–5.0 yr). These tissues were mainly from temporal lobectomies. Entire brains of 10 aborted fetuses were obtained from Advanced Bioscience Resources, Inc. (Alameda, CA). They were from fetuses 19.8 ± 1.8 wk old (range 17.5–24.0 wk).

Human brain tissue was prepared as previously described (18) and cultured in Iscove’s modified Dulbecco’s medium (Irvine Scientific, Santa Ana, CA) containing 10% non–heat-inactivated FCS (Gemini Bioproducts, Inc., Calabasas, CA) as well as 1-glutamine (2 mM) and gentamicin sulfate (50 μg/ml), both purchased from Irvine Scientific. Once cultures were established (1–4 wk), they were split at passage 1 and maintained in two variations of Iscove’s medium: 1) containing equivalent moles of recombinant vPE env proteins, WR, or mock-infected control supernatants. To control for any effects of vaccinia virus proteins, WR control supernatants were added to glial cells for the same length of time and at a concentration equal to the highest concentration of any of the vPE supernatants. After replacement of medium and an additional 2-h incubation, the glial cell supernatants were harvested. Therefore, the time designated for glial cell supernatant harvests for IL-1 and TNF-α is a total time of 4 h after initial exposure to env proteins or control supernatants. While other time points (8 h, 24 h) were examined, 4 h proved to be optimal. These glial cell supernatants contained no HIV-1 or vaccinia virus proteins as determined by analysis of Western blots using anti-gp160 or anti-gp41 and anti-vaccinia virus. This is probably due to the fact that the glial cells were washed after their 2-h exposure to supernatants from HeLa cells. For NO measurements, the incubation of glial cells with env proteins proceeded for 1 wk before the supernatants were harvested, which proved to be a peak production time point.

Treatment of Glial Cultures with Bacterially Produced Recombinant env Proteins and Heat-inactivated HIV-1 Virus. Recombinant HIV-1 env proteins (gp160, gp120, and gp41) and the control gag protein (p24) produced in bacteria were purchased from American BioTechnologies (Cambridge, MA) and used to stimu-
late glial cell cultures at 2 μg/ml. As described previously (18), HIV-1NL4-3 and HIVMN-SX were gradient purified, heat inactivated at 65°C for 45 min, and added to glial cells at a p24 concentration of 100 ng/ml. For total NO (NOx) production and IL-1/TNF-α/iNOS Northern blot analyses, stimulation with IL-1β (5 ng/ml) plus IFN-γ (70 ng/ml) (both cytokines from Immunex Corp.) was used as a positive control. With recombinant env proteins and heat-inactivated HIV-1 strains, stimulation of glial cells was performed for 4 h and 1 wk for assessment of IL-1/TNF-α and NO, respectively. As a control for the bacterially expressed recombinant envelope proteins, the gag protein of HIV-1, p24, was used as a negative control for the production of NO.

**HIV-1 Infection of Glial Cells.** Gradient-purified virus (18–20) from the T cell-tropic strain of HIV-1 (NL4-3) and the M0-tropic strains JR-FL and JR-CSF as well as a hybrid recombinant strain (NFN-SX) (18, 21) were used to attempt to infect human glial cultures. Glial cultures and control PBLs at 10^6 cells/culture in media with GCT or cytokines were exposed to 10^4–10^5 infectious U (300–500 ng/culture, p24 titer as measured by the HIV-1-specific ELISA [Abbott Laboratories, North Chicago, IL]). At this multiplicity of infection, as expected, maximal infection of the PBLs with the virus occurred at 1 viral DNA copy per 1,000 cells. After exposure to virus, 10% of the total cells was assayed by PCR. As fewer brain cells were exposed to virus, the relative amount of virus added per cell was ~50-fold higher for the brain-derived cells. In one experiment, tissue was processed into a cell pellet and then used in the same experiment. The nitrite (NO2-) plus nitrate (NO3-) production and IL-1, TNF-α, and NOx- assays were performed for 4 h and 1 wk for assessment of IL-1/TNF-α and NO, respectively. As a control for the bacterially expressed recombinant envelope proteins, the gag protein of HIV-1, p24, was used as a negative control for the production of NO.

**PCR Analysis of HIV-1 Entry and Infection in Glial Cells.** At the times designated above, DNA was recovered and subjected to 25 cycles of PCR amplification according to techniques previously described (18, 20). Quantitation was accomplished by analyzing in parallel a standard curve of cloned HIV-1 JR-CSF DNA linearized with EcoRI, which does not cleave viral sequences.

**Endotoxin levels of all reagents were measured by the limulus amebocyte lysate assay kit (BioWhittaker, Inc., Walkersville, MD).** Only those recombinant proteins, azidothymidine (AZT) or heat-inactivated viruses, live viruses, vPE, WR, and HeLa supernatants that had an endotoxin content of ≤10 pg/ml were used to treat the glial cells (19).

**Statistical Analysis.** The levels of IL-1, TNF-α, and NOx- produced by stimulation of glia were analyzed for significance by means of a multivariate analysis of variance (ANOVA). The paired Student’s t test was performed comparing IL-1, TNF-α, or NOx- activity in post facto analyses.

## Results

**Cellular Composition of Human Mixed Glial Cultures.** Markers for cell identification in fetal, neonatal, and adult cultures grown in two types of medium are shown in Table 1. There are no significant numbers of oligodendrocytes, endothelial cells, or neurons in these cultures. HLA-DR molecules were seen predominantly on astrocytes and not microglia. CD4 molecules were not present on fetal glia. We believe the minor population of CD4-positive cells in neonatal and adult brain cultures may be blood-borne macrophages or perivascular microglia (also blood-derived) contaminants and not parenchymal microglia. As enumerated by glial fibrillary acidic protein or α-LDL, there were equal proportions of astrocytes and microglia, respectively, in fetal, neonatal, and adult mixed glial cultures whether they were cultured in CYTO or GCT. Microglia were also identified by EBM-11 and RCA-1. EBM-11 identified fewer microglia than did α-LDL in fetal and neonatal cultures and disappeared entirely from microglia in adult cultures. RCA-1 identified all microglia and stained up to 50% of astrocytes in fetal and neonatal cultures, but not adult cultures (Table 1).

**Infection of and Pathology in Human Mixed Glial Cell Culture Exposed to HIV-1.** There are very few published studies on the infection of primary cultures of human glia, and the data are not always in agreement. Because of this, and because there is controversy as to the presence of CD4 on microglia in vivo and in vitro and whether it is the gp120...
Table 1. Human Mixed Glial Cultures: Cellular Composition; Percentage of Positive Cells

| Cell marker | Fetal | Neonatal | Adult |
|-------------|-------|----------|-------|
|             | CYTO  | GCT      | CYTO  | GCT      | CYTO  | GCT      |
| n = 8       |       |          | n = 9 |          | n = 4 |
| Astrocytes  |       |          |       |          |       |          |
| Glial fibrillary acidic protein | 40.5 ± 5.1 | 59.2 ± 15.1 | 55.5 ± 7.1 | 43.6 ± 7.3 | 40.0 ± 10.3 | 50.5 ± 3.8 |
| Microglia   |       |          |       |          |       |          |
| α-LDL      | 54.0 ± 15.3 | 37.5 ± 10.2 | 30.0 ± 3.5 | 39.0 ± 13.6 | 62.5 ± 10.1 | 56.7 ± 5.0 |
| EBM-11     | 29.3 ± 25.0 | 16.7 ± 6.1  | 18.3 ± 3.5 | 11.3 ± 2.0  | 0 ± 0  | 0.0 ± 0  |
| RCA-1      | 58.3 ± 4.3 | 71.3 ± 5.3 | 67.5 ± 10.3 | 66.7 ± 8.8 | 60.0 ± 1.3 | 63.5 ± 11.3 |
| Neurons     |       |          |       |          |       |          |
| NSE        | 0 ± 0  | 0 ± 0    | 6.7 ± 6.5 | 0 ± 0    | 0 ± 0  | 0.0 ± 0  |
| Oligodendrocytes |       |          |       |          |       |          |
| Galactocerebroside | 2.0 ± 2.0 | 0 ± 0 | 0.4 ± 0.4 | 0 ± 0 | 0 ± 0 | 0.0 ± 0 |
| Endothelial cells |       |          |       |          |       |          |
| FVIII       | 0 ± 0  | 0 ± 0    | 2.8 ± 2.0 | 0 ± 0    | 0 ± 0  | 0.0 ± 0  |
| Other       |       |          |       |          |       |          |
| HLA-DR      | 15.6 ± 5.0 | 4.3 ± 4.0 | 11.1 ± 3.5 | 6.0 ± 2.7 | 26.7 ± 20.0 | 0.0 ± 0 |
| CD4         | 0 ± 0  | 0 ± 0    | 4.0 ± 4.0 | 0 ± 0    | 6.7 ± 5.1 | 5.0 ± 5.0 |

EBM-11, CD68; NSE, neuron-specific enolase; FVIII, factor VIII; n, number of different brains cultured and stained; CYTO, IL-3, GM-CSF, M-CSF in Iscove’s medium; GCT, 10% GCT in Iscove’s medium. Means ± SD are shown.

receptor in the brain, we have examined the entry of HIV-1 into glial cells using PCR primers specific for either initiation (R/U5) or completion of reverse transcription (LTR/gag). This was done to rule out incomplete infection of glial cells (20) at 2, 3, 7, and 12 d after virus exposure. We compared GCT (a “black box” cocktail of cytokines)–grown cells to cells grown in a well-defined cytokine cocktail (CYTO, Table 1) that promotes survival and proliferation of microglia.

For PCR analyses, infection with heat-inactivated virus was performed in parallel with live virus. This control provides a background level for viral DNA contaminating the virus stocks. This contaminant DNA presumably arises from lysis of virions from infected cells during preparation of the stock. Regardless of the excess amount of virus used to infect glial cells, there was relatively little infection compared with PBLs. HIV-1 strains NL4-3 and JR-CSF never produced detectable infection over background in any culture. The more macrophage-tropic HIV-1JR-FL and recombinant NFN-SX strains produced an extremely low level of infection in four out of five fetal brains, using either fresh tissue or cultured cells. Cultured fetal cells were infected only minimally when in the GCT medium (Fig. 1 B). In contrast, we detected virus only rarely in neonatal tissue (only twice in 10 different brain cultures) and only if cells were in medium containing IL-3, GM-CSF, and M-CSF (CYTO, Fig. 1 B). Adult tissue, cultured either in CYTO or in GCT, was never detectably infected (Fig. 1 A, Table 2). The positive infections in PBL controls, which were analyzed within the first 20 h of infection such that no virus spread could occur, produced HIV-1 copy numbers in the hundreds to thousands, indicating the highly infectious nature of the virus stocks (Fig. 1, Table 2). Fresh preparations of fetal glial cells were infected at the same level as cultured fetal glia, suggesting that the culture conditions were not an explanation for low levels of HIV-1 infection. Similar data were generated whether R/U5 or LTR/gag primer pairs were used. When infection occurred, cultures were positive for infection by PCR analysis at 72 h. Culturing cells out to 12 d did not change these results (data not shown).

In spite of the low level of infection, a certain amount of morphological change occurred in fetal cultures exposed to virus. Fig. 2 demonstrates in vitro “pathology” in cultures that were not productively infected with the NFN-SX strain. GCT cultures displayed changes that were somewhat different from those in CYTO cultures, in that GCT cultures exposed to HIV-1 became gliotic. Astrocytes changed from an epitheloid to a spindly morphology (Fig. 2, days 2 and 12). Large clumps of both microglia and astroglia developed as determined by double staining (Fig. 2, days 2 and 12 and data not shown). The morphology of the mixed glia was not as dramatically altered in CYTO cultures (e.g., not as much gliosis). Nevertheless, monolayers did not grow in a confluent manner (Fig. 2, day 12), and
Figure 1. Quantitative PCR analysis of HIV-1 infection of human glial cell DNA from human glial cell cultures exposed to live HIV-1_{NF55X}. Cells were cultured with HIV-1 for 72 h in the presence or absence of AZT, or they were cultured with heat-inactivated (HI) virus. Stimulated PBLs were used as a positive control. Control cells were harvested within 20 h of infection, before the time virus spread would occur, to indicate levels of infectious virus in the inoculum. As cultured cells were exposed to equal amounts of virus and 10% of treated cells were analyzed, one would expect equal levels of viral DNA in brain versus PBL if the infectivity of the cells was identical. AZT has minimal effect on signals generated by the R/U5 primer pair since it does not significantly inhibit initiation of reverse transcription, as previously shown (20). The primer pair M667/M661 specific for the LTR/gag region of HIV-1_{Jr,FL}, which results in a 200-bp amplified DNA fragment, detects complete reverse transcripts. The DNA primer pair specific for the R/U5 region of HIV-1_{Jr,FL}, which results in a 140-bp amplified fragment, was used to detect initiation of reverse transcripts. Use of the R/U5 primer pair is more sensitive than LTR/gag. The DNA standards used for HIV were cloned HIV-1_{JR,F}.

Table 2. Human Mixed Glial Cell Cultures: Infection with HIV-1 Assessed by PCR

| Medium          | Fetal | Neonatal | Adult |
|-----------------|-------|----------|-------|
|                 | CYTO  | GCT      | 0     | CTYO  | GCT      | CTYO  | GCT      |
| Glia, fresh     | 0     | 10       | 10    |       |          |       |          |
| Glia, cultured  | 0 ± 0 | 7 ± 5*   | 30 ± 10* | 0 ± 0 | 0 ± 0    | 0 ± 0 | 0 ± 0    |
| PBL             | 300 ± 100 | 450 ± 100 | 400 ± 50 |      |          |       |          |

Data represent virion copies/10^6 cells.

*Data represent the mean ± SD of all five brains where four positive infections occurred out of the five brain samples.

†Data represent the mean ± SD of the two positive infections of 10 brain samples.

n, number of different brains cultured and exposed to HIV-1_{NF55X} for PCR analysis. R/U5 primer pair was used; CYTO, IL-3, GM-CSF, M-CSF in Iscove’s medium; GCT, 10% GCT in Iscove’s medium. 0, Iscove’s medium without GCT or CYTO. Means ± SD are shown.
microglia were increased in numbers in these cultures (Fig. 2, day 7).

IL-1 and TNF-α Induction in Human Mixed Glial Cultures. Neither human microglia nor astrocytes produce as much IL-1 or TNF-α when cultured as isolated subsets as they do when cultured together, where they synergize to produce maximal cytokines (28, 29). Because coculturing both cell types is more relevant to the inflammatory milieu in vivo, we did not separate them into subpopulations for induction of cytokines or NO. Figs. 3 and 4 demonstrate that HIV-1 env proteins and heat-inactivated virus induce IL-1 and TNF-α proteins and mRNA in human glial cell cultures, and that the age of tissue and culture conditions make a difference in the amount of cytokines induced.

Fig. 3 demonstrates IL-1 and TNF-α production in cultured glia. In general, in cultures with significant IL-1 induction, fetal cultures produced significantly more IL-1 in response to vPE16, -17, -20, and -21, and neonatal cultures produced significantly more IL-1 in response to vPE16, -17, and -21 in CYTO medium than in GCT medium. Likewise, fetal cells stimulated with vPE17, -18, and -12 produced significantly more IL-1 in GCT medium than neonatal cells. Fetal CYTO cultures produced significantly more IL-1 in response to vPE16, -20, and -12 than did adult cells and to vPE18, -8, and -20 than did neonatal cells. Adult glia responded similarly whether in CYTO or GCT medium. After vPE16, -17, and -18 stimulation in GCT medium, adult glia produced significantly more IL-1 than fetal cells.

Compared with internal controls (medium, HeLa, or WR), the best inducers of IL-1 in fetal and neonatal CYTO and adult GCT cultures were vPE16 and/or the heat-inactivated, MØ-tropic HIV-1 strain NFN-SX. In fetal CYTO cultures, vPE16 was significantly better than vPE17, -18, -8, -20, -21, and -12 at IL-1 induction. In neonatal CYTO cultures, vPE16 was significantly better than vPE18, -8, -20, -21, and -12. No such differences were seen in GCT medium in fetal and neonatal cultures. In contrast, in adult cultures, no differences in any vPE induction except vPE21 were seen in CYTO medium, while in GCT, vPE16, -17, -18, and -12 induced significantly more IL-1 than vPE8, -20, and -21 (Fig. 3). vPE21 induced the lowest levels of IL-1 in all cultures. The MØ-tropic strain was significantly better than the T cell-tropic strain at IL-1 induction in fetal, neonatal, and adult cells cultured in CYTO. Recombinant gp41 induced a small but significant IL-1 response compared with the medium control.

Fetal and neonatal cells behaved differently from adult cells.
Figure 3. IL-1 and TNF-α induction in human mixed glial cells. Mixed glial cultures (50% astrocytes, 50% microglia) from different ages of human brain tissue grown under different conditions (GCT, CYTO) were exposed to medium alone (MED), control vaccinia virus-infected HeLa cells (WR), recombinant vaccinia virus env proteins produced in HeLa cells (vPE), a recombinant env protein produced in bacteria (gp41), and heat-inactivated, T cell–tropic (NL4-3, NL) and M0–tropic (NFN-SX, SX) HIV-1 virions. Supernatants were harvested at 4 h for assessment of IL1 and TNFα production. Means ± SD are shown for different numbers of brains cultured: fetal (n = 10), neonatal (n = 4), and adult (n = 4). IL-1 responses were as follows. Fetal: CYTO vs GCT: vPE16, -17, -20, P < 0.01; fetal vs neonatal in CYTO: vPE17, -18, -12, P < 0.01; fetal vs neonatal in CYTO: vPE18, -8, -20, P < 0.05; fetal vs adult in CYTO: vPE16, -18, -20, -12, P < 0.05; CYTO vPE16 vs vPE17, -18, -8, -20, -21, -12, P ≤ 0.001; CYTO SX vs NL, P ≤ 0.02. Neonatal: CYTO vs GCT: vPE16, -17, -21, P < 0.001; CYTO vPE16 vs vPE18, -8, -20, -12, P ≤ 0.01; CYTO SX vs NL, P ≤ 0.02. Adult: adult vs fetal in GCT: vPE16, -17, -18, P ≤ 0.01; adult vs neonatal in GCT: vPE16, -17, -18, -8, -12, -21, P ≤ 0.001; GCT vPE16, -17, -18 vs vPE8, -20, -21, P ≤ 0.05; CYTO SX vs NL, P ≤ 0.01. All gp41, NL, SX vs MED, P ≤ 0.01. All vPE vs WR, P ≤ 0.001. All other comparisons were not significant. TNF-α responses were as follows. Fetal: CYTO or GCT: vPE16, -17, -18, -12 vs vPE8, -20, P ≤ 0.01; CYTO or GCT SX vs NL, P ≤ 0.001. Neonatal: neonatal vs fetal or adult in GCT or CYTO: vPE18, -8, -21, P ≤ 0.001; GCT vPE16, -18 vs vPE8, -20, -21, P ≤ 0.01. Adult: GCT vPE16 vs vPE8, -20, -21, P ≤ 0.01; GCT vPE17 vs vPE20, -21, P ≤ 0.05. The multivariate ANOVA and paired Student’s t test analyses were used for statistical significance.

cells in response to IL-1 induction by envelope proteins given that CYTO medium allowed for more IL-1 and TNF-α production than did GCT. Adult cells did better in GCT medium than fetal or neonatal cells. Epitopes in gp41 are capable of inducing IL-1, as evidenced by stimulation with recombinant gp41 protein and by the power of vPE16 to induce IL-1 better than truncation vPEs with deletions in gp41. Removal of the V3 loop (vPE21) significantly reduced IL-1 induction. In fetal and neonatal cultures, the conformational interaction of gp120 and gp41 may be important, since vPE12 (an uncleavable gp160) gave a significantly lower IL-1 induction than did vPE16.

Fig. 3 also demonstrates the induction of TNF-α in fetal, neonatal, and adult glial cultures. In contrast to medium/cofactor–dependent differences seen in IL-1 induction (with a single exception of fetal in GCT induction by NFN-SX), TNF-α induction was the same in GCT and CYTO medium in fetal, neonatal, and adult cultures. In response to vPE18, -8, and -21, neonatal glial cells produced significantly more TNF-α than did adult or fetal cells, which were not significantly different from each other.
other at any data point. As with IL-1, adult, neonatal, and fetal cultures specifically produced the same amount of TNF-α in response to heat-inactivated virus and recombinant gp41.

In fetal cultures, vPE16, -17, -18, and -12 induced the same levels of TNF-α but significantly more than was induced by vPE8 and -20, which produced similar TNF-α. This suggests that gp41 is important and the V3 loop is not important in TNF-α production. Likewise, in neonatal and adult cultures, removal of all gp41 epitopes (as in vPE8, -20, -21) significantly reduced TNF-α in contrast to what was seen for IL-1. vPE21 did not induce significant TNF-α in any cultures. In contrast to IL-1 induction, gp41 stimulated as much TNF-α as did vPE16 and heat-inactivated virus induction. These data suggest a more important role for gp41 in TNF-α than IL-1 induction. In fetal cultures, the MO-tropic strain induced significantly more TNF-α than did the T cell–tropic strain; there was no tropism in TNF-α induction in neonatal or adult cultures.

To determine whether the induction of IL-1 and TNF-α was being regulated at the mRNA level, Northern blot analyses of IL-1 and TNF-α mRNA from fetal cultures before and after env protein stimulation were performed compared with the housekeeping cyclophilin gene. In Fig. 4 A, it can be seen that the negative controls, medium, or WR vaccinia virus did not induce IL-1 or TNF-α mRNA. Likewise, vPE21, which did not induce protein, also did not induce mRNA. The positive control IFN-γ/IL-1 was a strong inducer of both cytokine mRNAs in either medium (GCT or CYTO). For IL-1, vPE16 was a better inducer than vPE8 or vPE20, a difference that was greater in CYTO than GCT in parallel with protein data (Fig. 3). The differences among vPE16, -18, and -20 or between CYTO and GCT were not as pronounced in TNF-α induction.

**NO Induction in Fetal Human Mixed Glial Cultures.** Figs. 4 B and 5 demonstrate the effects of bacterially produced recombinant env proteins and truncated but glycosylated recombinant env proteins expressed in HeLa cells to induce NO synthase and NO− production in fetal glial cultures. Glial cells produce more NO in either GCT or CYTO media after stimulation with env proteins compared with the controls (medium or HIV-1 p24 for bacterially produced recombinants or WR for vaccinia virus recombinants). Medium alone and HeLa cell supernatants gave no induction. With the exception of gp160 in GCT medium, culturing cells in GCT or CYTO did not influence NO production. The NO response to vPE env proteins was lower than that to bacterially produced env proteins, which induced responses not significantly different from each other or the positive control IFN-γ/IL-1. vPE20 and -21 did not induce significant iNOS or NO− production, indicating that the V3 loop and amino terminus of gp120 are not important (Figs. 4 B and 5). The overall pattern of NO response thus resembled the TNF-α response to HIV-1 env (compare Figs. 3 and 5).

**Discussion**

This is the first report to our knowledge showing that envelope proteins of HIV-1 are capable of inducing cytokines and NO in normal human glial cultures. We and others have demonstrated NO production by rat microglia (13, 23, 25) and a role for TNF-α and NO in white matter damage, including toxicity for oligodendrocytes and inhibition of myelin gene expression (26, 27). We have suggested that NO may damage oligodendrocytes without killing them. This is particularly relevant to CNS AIDS, where oligodendrocytes are only destroyed very late in disease (1) but myelin damage is seen, especially in spinal cord, even in the presence of oligodendrocytes (28). NO may also be of importance in neuronal toxicity and loss in CNS AIDS (1). Although the literature is at variance over how neuronal toxicity is produced in vitro, most studies show indirect toxic events as opposed to direct infection (29 and references therein).

We hypothesize that IL-1, TNF-α, and NO production after env protein stimulation are interrelated and result in the gliotic pathology seen in vivo and as shown in vitro in this study. In vivo in AIDS brains, astrocytes undergo gliosis because of hypertrophy (30); in these circumstances, glial fibrillary acidic protein positivity by immunohistochemistry increases (1, 30). IL-1, TNF-α, and NO cause a gliotic response in both microglia and astrocytes in vitro (14, 31). Thus, we presume env proteins are indirectly in-
duc ing the microgliosis shown in vitro in this study. While NO may cause glial death, we would predict little or no other perceptible toxic effect in these cultures. We and others have shown that NO inhibits mitochondrial function in astrocytes, but it does not kill them. NO has virtually no toxic effect on microglia (23, 27).

If the production of NO in our cultures is the indirect result of IL-1 and TNF-α production or a direct effect of gp120/gp41 on NFkB, it is not clear why there is a rather long interval between IL-1 and TNF-α production (peak 4-24 h) and peak NO production (3-7 d). Posttranslational regulation of iNOS may be an important component in the delay in NO production. This is currently being examined.

As we have suggested in these studies, HIV-1 need not infect glial cells to contribute to pathology in the CNS. While it is clear that HIV-1 infects CNS microglial cells in vivo, productively infected cells are rare in the brains of pediatric AIDS patients. The in vivo infections are latent or defective (1-3, 32, 33). Even the culturing of microglia from infected pediatric brain tissue requires 30 d before viral replication is readily apparent, indicating the extremely slow virus life cycle or poor infectivity leading to slow spread of infection (34). In vitro infection of astrocytes from fetal brain results in a transient, partially defective viral replication (7-9). In vivo, astrocytes are not productively infected (1) but nef protein is expressed in a small proportion of astrocytes in pediatric AIDS brain tissues (35). A restricted or latent infection in glia is therefore thought to lead to indirect but extensive neuropathogenesis.

While several reports claim microglia in brain tissue and in tissue culture have CD4 on their surface (10, 34), which contributes to HIV-1 infection (10), many other studies have failed to detect CD4 on parenchymal microglia in fetal or adult brain from normal or HIV-1–positive brain tissue (11, 12, 36, 37), nor has CD4 been seen to be inducible on microglia in vitro (37). It is thought that the CD4+ cells in brain are blood-derived perivascular CNS macrophages (11, 36). Since we document a significant number of microglia in our cultures (which are CD4−), either the Watkins study (10) and follow-up studies from the same laboratory were demonstrating infection of blood-borne, perivascular brain macrophages and not microglia, or other culture conditions in the Watkins study allowed for CD4 expression and/or infection. The infection we achieved in fetal cultures, while significantly above controls and on a fairly consistent basis, was extremely low compared with PBL controls. The fetal tissue we used was taken during the second trimester and was therefore older than that in one study (11) but similar to that in another (12). Nevertheless, the level of infection in our study was significantly lower than that reported by Lee et al. (12). Additionally, the inability to infect human glia in vitro and the differences seen in TNF-α, IL-1, and NO production when cells are in different medium conditions suggest that the in vitro cofactors are not identical to in vivo cofactors, or that in vivo infection of glia is indirectly achieved by fusion of infected blood monocytes with brain cells.

In summary, this study reconfirms two previous studies on rat glial cells demonstrating a functional cytokine response to non-CD4 epitopes in gp120 and to the amino terminus of gp41 (18, 19). IL-1, TNF-α, and iNOS can be induced at the mRNA level by env proteins and HIV-1 virus in the absence of infection. Their induction is induced and regulated differently, given that media with different cofactors and different env domains give different IL-1 or TNF-α responses. The responses in adult, neonatal, and fetal glial cultures are also different, even though the cellular composition of the cultures appears to be identical. The V3 loop and carboxy terminus of gp120 and to a lesser extent gp41 are important for IL-1. The V3 loop of gp120 is less important and gp41 is more important in TNF-α than in IL-1 induction. While the macrophage-tropic strain of HIV-1 tends to be a better inducer of cytokines, a significant IL-1 and TNF-α response occurs with a T cell–tropic strain of HIV-1. Further mapping of distinct sequences within gp120 and gp41 that are responsible for TNF and IL-1 production and the role of these cytokines in NO induction is part of the ongoing research in this lab. Elucidation of these interactions will provide a basis for designing therapeutic interventions to use in treating CNS AIDS.

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