

Cytokines and Arachidonic Metabolites Produced during Human Immunodeficiency Virus (HIV)-infected Macrophage-Astroglia Interactions: Implications for the Neuropathogenesis of HIV Disease

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Summary

Human immunodeficiency virus (HIV) infection of brain macrophages and astroglial proliferation are central features of HIV-induced central nervous system (CNS) disorders. These observations suggest that glial cellular interactions participate in disease. In an experimental system to examine this process, we found that cocultures of HIV-infected monocytes and astroglia release high levels of cytokines and arachidonate metabolites leading to neuronotoxicity. HIV-1_{ADA}-infected monocytes cocultured with human glia (astrocytoma, neuroglia, and primary human astrocytes) synthesized tumor necrosis factor (TNF- α) and interleukin 1 β (IL-1 β) as assayed by coupled reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assay, and biological activity. The cytokine induction was selective, cell specific, and associated with induction of arachidonic acid metabolites. TNF- β , IL-1 α , IL-6, interferon α (IFN- α), and IFN- γ were not produced. Leukotriene B₄, leukotriene D₄, lipoxin A₄, and platelet-activating factor were detected in large amounts after high-performance liquid chromatography separation and correlated with cytokine activity. Specific inhibitors of the arachidonic cascade markedly diminished the cytokine response suggesting regulatory relationships between these factors. Cocultures of HIV-infected monocytes and neuroblastoma or endothelial cells, or HIV-infected monocyte fluids, sucrose gradient-concentrated viral particles, and paraformaldehyde-fixed or freeze-thawed HIV-infected monocytes placed onto astroglia failed to induce cytokines and neuronotoxins. This demonstrated that viable monocyte-astroglia interactions were required for the cell reactions. The addition of actinomycin D or cycloheximide to the HIV-infected monocytes before coculture reduced, >2.5-fold, the levels of TNF- α . These results, taken together, suggest that the neuronotoxicity associated with HIV central nervous system disorders is mediated, in part, through cytokines and arachidonic acid metabolites, produced during cell-to-cell interactions between HIV-infected brain macrophages and astrocytes.

The central nervous system (CNS)¹ is a major reservoir for HIV (1–5), and virus is expressed almost exclusively in cells of macrophage lineage (brain macrophages, microglia,

and multinucleated giant cells) (6–10). Productive HIV replication in brain macrophages and microglia often predict neurological disease (4, 5). In affected tissue up to 15% of brain macrophages express HIV gene products (6–10). Nevertheless, the role of these HIV-infected cells in disease pathogenesis remains poorly understood.

Several studies demonstrate entry of virus into the CNS

¹ Abbreviations used in this paper: CNS, central nervous system; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MOI, multiplicity of infection; NSE, neuron-specific enolase; RT, reverse transcriptase.

early after infection, either during the acute seroconversion reaction or during subclinical infection (11–15). However, exactly how HIV enters the brain and preferentially infects macrophages are areas of intense debate. Virus-infected brain macrophages may originate from an expansion of latently infected monocytes that carry HIV into the brain (the “Trojan horse” hypothesis) and later produce virus (16). Alternatively, virus may penetrate the brain through a disrupted blood-brain barrier by infected T cells or as free viral particles. In either case the results are identical: selective productive infection of brain macrophages and microglia. Whether these HIV-infected brain macrophages induce disease through metabolic, immune, and/or viral-induced mechanisms is critical to our understanding of HIV neuropathogenesis (17). These issues are made ever more apparent as several reports suggest that low-level infection of neurons and glia can produce neurological impairment during HIV infection (18–20). The ultimate approaches to therapy would vary dependent upon discovered pathogenic mechanisms for CNS injury.

Proposed theories of CNS dysfunction abound. These include: coexistence of opportunistic CNS infections (21, 22), secretory toxic factors from infected monocytes, gp120-mediated neuronal growth factor blockade or killing, and neurotoxicity by HIV *tat* or other viral regulatory components. All or any of these mechanisms may result in cytotoxic effects in neurons and/or oligodendrocytes (23–25). For example, gp120 may antagonize normal vasoactive intestinal peptide (VIP-ergic) function in brain (23) or be directly toxic to neurons. Studies show that gp120 can induce neuronotoxicity by increasing free Ca^{2+} levels in cultured neurons and is prevented by Ca^{2+} channel antagonists (24).

Recent reports suggest that brain dysfunction may be related to cell-encoded toxins generated from virus-infected macrophages (26, 27). Secretory products from HIV-infected cells may alter neuronal viability, damage myelin, or stimulate neurotransmitters resulting in neuronal dysfunction. Indeed, macrophages play important roles in steady-state immune and tissue function. The regulatory role of macrophages occurs through the release of numerous secretory molecules made under a variety of physiologic conditions. Changes in the secretion or release of certain of these mediators may lead to disease. In support of this idea are recent studies demonstrating that disordered secretion of one or more cellular factors from HIV-infected macrophages produces neuronal death in vitro (26, 27). In one report, HIV-infected U937 cells, a myelomonocytic cell line, released toxic factors that destroyed cultured chick and rat neurons (27). The monocyte-produced neurotoxin(s) were heat stable and protease resistant, and acted by way of *N*-methyl-D-aspartate (NMDA) receptors. The studies suggested that HIV-infected macrophages and microglia in brain continuously disrupt neurologic function leading to cognitive CNS dysfunction. However, two recent reports failed to confirm these observations (28, 29). One study (29) demonstrated neurotoxicity only after cell-to-cell contact between HIV-infected monocytic and human neural cells. In this and a study from our own laboratory (28) investigators found no morphological alterations of neurons exposed to HIV-1-

infected monocyte fluids. Thus, if the macrophage plays a role in virus-induced neuropathology it may act through cell-to-cell interactions with neurons and glia to produce CNS tissue damage (29). Perhaps this occurs through cytokine and/or other neuronotoxic factor release during cell-to-cell contact. Indeed, similar mechanisms are operative for cytokine induction in peripheral blood. For example, IFN- α is induced during cell-to-cell interactions with HIV-infected macrophages and PBMC (30). Similarly, neuronotoxins could be produced from glia during cell-to-cell interactions with infected brain macrophages. To investigate this possibility we recovered culture fluids from cell mixtures of virus-infected monocytes and astroglia. Assay of fluids after cocultivation showed high levels of arachidonic acid metabolites, cytokines, and neuronotoxic activity.

Materials and Methods

Isolation and Culture of Monocytes and Human Neural Cells. Monocytes were recovered from PBMC of HIV and hepatitis B-seronegative donors after leukapheresis and purified by countercurrent centrifugal elutriation. Cell suspensions were >98% monocytes by criteria of cell morphology in Wright-stained cytosmears, by granular peroxidase, and by nonspecific esterase. Monocytes were cultured as adherent monolayers (10^6 cells/ml in 24-mm plastic culture wells) in DMEM (Sigma Chemical Co., St. Louis, MO) with 10% heat-inactivated AB⁺ human serum, 50 μ g/ml gentamicin, and 1,000 U/ml highly purified (<0.01 ng/ml endotoxin) recombinant human macrophage MCSF (FAP-809; Cetus Corp., Emeryville, CA) (31).

Human brain tumor-derived cell lines were obtained from the following sources: U251 MG from D. Bigner (32), U373 MG from B. Westermarck (33), and SK-N-MC (34) and H4 (HTB 148) (35) from the American Type Culture Collection (ATCC; Rockville, MD). The cells were grown as adherent monolayers in DMEM (Sigma Chemical Co.) with 10% heat-inactivated FCS and 50 μ g/ml gentamicin. Human endothelial cells were a gift from P. I. Lelkes (36). Cell lines were fully characterized to their cell origins (37, 38). Primary human astrocytes were prepared from second-trimester human fetal brain tissue obtained from elective abortions (performed in full compliance with both National Institutes of Health and University of Rochester guidelines). Brain tissue composed of telencephalon with both cortical and ventricular surfaces was dissected in cold HBSS with HEPES and 50 μ g/ml gentamicin, then transferred to 20 ml ice-cold (4°C) DMEM/F12 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated FCS. The tissue was mechanically dissociated by teasing through a Nitex bag with a glass pestle. Cells were resuspended in media and filtered through a 230- and then 140- μ m sieve. The cell suspension was centrifuged, washed twice in media, then plated in DMEM containing 10% FCS and 50 μ g/ml gentamicin into 75-cm² tissue culture flasks (Corning, Corning, NY) at a cell density of 2×10^5 cells/ml. Media was exchanged every 3 d. Nonadherent microglia and oligodendrocytes were removed by gentle agitation and circular shaking of cultured cell preparations 10 d after plating. The purity of the astrocyte cultures was $\geq 95\%$ by immunostaining for glial fibrillary acidic protein (GFAP) (39). Cells were cultured as adherent monolayers in DMEM (Sigma Chemical Co.) with 10% heat-inactivated FCS (Sterile Systems, Inc., Logan, UT), 20 μ g/ml gentamicin, and 1% glutamine. All culture reagents were screened and found negative for endotoxin contamination.

HIV Infection of Target Cells. Adherent monocytes cultured for 7 d were exposed at a multiplicity of infection (MOI) of 0.01 infectious virus/target cell to ADA, or 24 monocyte tropic HIV-1 strains (40). All viral stocks were tested and found free of mycoplasma contamination (Gen-probe II; Gen-probe Inc., San Diego, CA). Culture medium was half-exchanged every 2–3 d. Reverse transcriptase (RT) activity was determined in replicate samples of culture fluids added to a reaction mixture of 0.05% NP-40 (Sigma Chemical Co.), 10 µg/ml poly(A), 0.25 U/ml oligo(dT) (Pharmacia Fine Chemicals, Piscataway, NJ), 5 mM dithiothreitol (Pharmacia Fine Chemicals), 150 mM KCl, 15 mM MgCl₂, and ³H-dTTP (2 Ci/mmol; Amersham Corp., Arlington Heights, IL) in pH 7.9 Tris-HCl buffer for 24 h at 37°C. Radiolabeled nucleotides were precipitated with cold 10% TCA and washed with 10% TCA and 95% ethanol in an automatic cell harvester (Skatron Inc., Sterling, VA) on glass filter discs. Radioactivity was estimated by liquid scintillation spectroscopy (41).

Chemical Reagents. Dexamethasone was purchased from Sigma Chemical Co. and indomethacin and nordihydroguaiaretic acid (NDGA) from Cayman Chemical Co., Ann Arbor, MI. All reagents were dissolved in ethanol and diluted with complete macrophage media. Final ethanol concentration in cell cultures was ≤0.1%.

Quantitations of Cytokine Activity. Culture fluids from control and HIV-infected monocytes were analyzed by ELISA for the human cytokines TNF-α, IL-1α, IL-1β, and IL-6 (Quantikine Immunoassay; Research and Diagnostics Systems, Minneapolis, MN). IFN activity in culture fluids was assayed by inhibition of cytopathic effects induced by vesicular stomatitis virus (VSV) in MDBK cells (42). TNF bioactivity was performed according to standard procedures (43). Briefly, the murine L929 cell line was propagated in DMEM (Sigma Chemical Co.), 5% FCS, 1% glutamine, and 20 µg/ml gentamicin. Cells were retrieved in log phase and placed (0.5 × 10⁵/well) in 96-well plates (Costar) with actinomycin D. Culture fluids were inoculated into cell monolayers, and degree of cell lysis was determined by crystal violet staining after a 24-h incubation.

Coupled RT-PCR Detection of Cytokine and HIV-specific RNA. Levels of cytokine RNAs were estimated after RT with antisense primers and PCR amplification of the cDNA transcripts. The mRNA for the cellular enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), served as an internal control to allow analysis and comparison of RNA species between different samples. Briefly, 2.0 µg total cellular RNA in 0.025 ml was mixed with 0.3 µg of the antisense primers for GAPDH, TNF-α, TNF-β, IL-1α, IL-1β, IL-6, IFN-α, IFN-γ, and IFN-β (44). Table 1 lists the primer sequences used in these studies. The mixture was heated at 70°C for 5 min, cooled on ice, and treated with 500 U of Moloney murine leukemia virus RT (Bethesda Research Laboratories, Bethesda, MD) and 0.5 mM each of all four deoxynucleotide triphosphates. RT reactions were at 37°C for 15 min, then stopped by heating at 95°C for 10 min. For PCR amplification of the cDNA products, reaction mixtures were divided into equal aliquots and mixed with 0.5 µg sense and antisense primers, 0.5 mM deoxynucleotide triphosphates, and 2 U Amplitaq (Cetus Corp., Emeryville, CA). The products of 25 cycles (1.5 min, 94°C; 1.5 min, 50°C; and 2.0 min, 72°C) were analyzed by Southern blot hybridization. The oligo-nucleotides were synthesized on DNA synthesizer (Applied Biosystems, Inc., Foster, City, CA) and checked for purity by polynucleotide kinase labeling and sequence gel analysis. Oligonucleotides were typically 95% pure.

Fetal Rat and Human Brain Cortical Explant Cultures. Fetal Sprague-Dawley rat (15-d gestational age) forebrains were dissociated

by titration into a single cell suspension and adjusted to 10⁶ viable cells/ml. Cells were plated in poly-L-lysine-treated plastic culture wells in a 1:1 mixture of Eagle and Ham's F12K medium with 50 U/ml penicillin, 50 µg/ml streptomycin, 600 µg/ml glucose, 10% horse serum, and 10% FCS (45). Alternatively, cells were plated into N5 medium supplemented with 5% horse serum fraction (46). After 5 d, cultures were treated with 10 µM cytosine arabinoside (Ara-C) (Sigma Chemical Co.) for 48 h to deplete proliferating astrocytes, fibroblasts, and microglial cells (29). The composition of these Ara-C-treated cultures at day 10 was 70–85% neurons by neuron-specific enolase (NSE; Dako Corp., Carpinteria, CA), 10–15% microglia by latex-bead phagocytosis and rat OX-6 staining, and <5–10% astrocytes by morphology and glial fibrillary acidic protein (GFAP; Dako Corp.) staining. Cells were treated with conditioned media from cell cultures for 1–7 d and analyzed for neurotoxicity.

Quantitations of Neuronal Cell Growth and Survival. The metabolic activity and number of viable cells/culture were assessed by conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) bromide, and color intensity was measured at OD_{490 nm} (47, 48). Cell morphology in neuronal cultures depleted of glial cells was examined under phase-contrast microscopy or after fixation with 80% methanol and Wright-Giemsa stain. Morphologic changes in these neuron-enriched cell cultures correlated directly with MTT levels and were scored as 0 (no neuritic outgrowth), + (dendritic outgrowth 2–4 perikaryons distance in ≥50% of cells/field), or ++ (dendritic outgrowth 4–8 perikaryons distance in ≥50% of cells/field). Generally, 100 cells in four fields/culture were examined on successive days. In some studies, identical microscopic fields were photographed at serial intervals to decrease sampling variability.

Analysis of [³H]Arachidonic Acid Metabolites Released by HIV-infected Monocytes and Cocultures of HIV-infected Monocytes and Astroglia. HIV-infected or control uninfected monocytes (5 × 10⁶ cells/ml) were cultured in 35-mm wells (Costar) in media containing 10% AB⁺ human sera and [³H]arachidonic acid (1.0 mCi/ml; 1 Ci = 37 GBq; American Radiolabeled Chemicals Inc., St. Louis, MO) added to the cultures for 18 h. HPLC procedures followed previously published methods (49, 50). A brief description of the protocol used in these experiments is outlined below.

The monocytes were washed three times with DMEM in 1% BSA (Sigma Chemical Co.) then incubated for 20 s to 180 min with or without equal numbers of U251 MG astroglial cells. The reaction was stopped by the addition of 10 µl formic acid and 25 µl butylated hydroxytoluene in methanol, and the samples were placed on dry ice. Supernatant fluids were removed and the cells scraped after the addition of 500 µl of HPLC-grade water. The cell lysate was combined with the culture supernatants from each well and placed into 10-ml polypropylene centrifuge tubes. The fractured cell-supernatant mixtures were centrifuged at 400 g for 5 min and the clarified supernatants stored under argon at –70°C in 4-ml dram vials. Before analysis, a 2-ml thawed sample was adjusted to pH 4.0 with 22 M formic acid and microcentrifuged for 1 min. The internal standard mix was added to the cell lysate. It contained hydroxyeicosadienoic acid (for spectrophotometric verification of elution position accuracy) and [¹⁴C]eicosatrienoic acid (for quantitation of sample recovery and inter/intrasample comparisons). DPMs of each sample were adjusted based on recovered [¹⁴C]eicosatrienoic acid. A C18 Sep-Pak cartridge (Waters Associates, Milford, MA) was activated by placing 4 ml of HPLC-grade methanol through the cartridge. The C18 Sep-Pak was washed with 10 ml of HPLC-grade water. The sample was applied to the C18 Sep-Pak cartridge (Waters Associates) followed by a 2.5-ml

wash, and arachidonic acid metabolites were quantitatively eluted with a mixture of 85% acetonitrile and 15% methanol. The eluant was concentrated and dried with a Speed-Vac concentrator (Savant Instruments, Inc., Farmingdale, NY). The arachidonic acid metabolites were then dissolved in methanol and transferred to HPLC vials (National Scientific Co., Lawrenceville, GA) for injection. The arachidonic acid metabolites extracted were then injected onto a reverse-phase C-18 column using an analytical HPLC system (Beckman Instruments, Inc., Palo Alto, CA). Spectrophotometric analyses were performed with a UV detector (166; Beckman Instruments, Inc.). 1-min fractions were collected during a 96-min

chromatography run, and total DPM was determined in each fraction. A second separately collected elution was performed on the Sep-Pak that quantitatively removed platelet-activating factor (PAF). The eluate was dried and analyzed using a quantitative RIA kit (Amersham Corp., Arlington Heights, IL).

Results

No Evidence for Neuronotoxic Activity in Culture Fluids of HIV-infected Monocytes or Glial Cells. Monocytes and glia (U251 MG, U373 MG, and H4 HTB 148) cells were infected with

Table 1. Nucleotide Primers in Coupled RT-PCR Detection of Cytokines

Amplification product size	Nucleotide position	Primer	Sequence
TNF- α 237 bp	503-527	Sense	GAGCTGAGAGATAACCAGCTGGTG
	740-716	Antisense	CAGATAGATGGGCTCATACCAGGG
	588-608	Probe	CCCTCCACCCATGTGCTCCTC
TNF- β 147 bp	238-258	Sense	AACATGACCCTCCTAGACCA
	385-366	Antisense	TCCCTGGAAGTACCTCCTCA
	341-360	Probe	GCAATTAGCAGCCCTGCACT
IL-1 α 201 bp	435-459	Sense	TTCGAGCCAATGATCAGTACCTCA
	636-615	Antisense	TTTGGGTATCTCAGGCATCTCC
	481-501	Probe	GATGAAGCAGTGAAATTTGAC
IL-1 β 179 bp	480-500	Sense	AAAAGCTTGGTGATGTCTGG
	659-638	Antisense	TTTCAACACGCAGGACAGG
	549-567	Probe	ATGGAGCAACAAGTGGTG
IL-6 159 bp	317-337	Sense	GTGTGAAAGCAGCAAAGAGGC
	476-455	Antisense	CTGGAGGTAATCTAGGTATAC
	399-420	Probe	GGATTCAATGAGGAGACTTGC
IFN- α 274 bp	240-259	Sense	TCCATGAGATGATCCAGCAG
	514-492	Antisense	ATTTCTGCTCTGACAACCTCCC
	433-454	Probe	AAATACTTCCAAAGAATCACT
IFN- β 186 bp	343-364	Sense	GATTCATCTAGCACTGGCTGG
	529-509	Antisense	CTTCAGGTAATGCAGAAATCC
	379-400	Probe	GAGAACCCTCCTGGCTAATGTC
IFN- γ 168 bp	372-391	Sense	GCATCCAAAAGAGTGTGGAG
	540-521	Antisense	GACAGTTCAGCCATCACTTGG
	463-482	Probe	GACTAATTATTCGGTAAGTACTGAC
GAPDH 195 bp	199-217	Sense	CCATGGAGAAGGCTGGGG
	394-374	Antisense	CAAAGTTGTCATGGATGACC
	280-299	Probe	CTAAGCAGTTGGTGGTGCA

HIV-1_{ADA} at an MOI of 0.01. Culture fluids from the HIV-infected and control uninfected cells were half-exchanged at 2–3-d intervals then added, 14 d after plating and 7 d after infection, to rat brain explant and/or SK-N-MC human neuroblastoma cells. The composition of fetal rat brain cultures at the time of experimental inoculation (day 10 of culture) was 70–85% neurons (neurofilament and neuron-specific enolase-positive cells), 10–15% microglia (OX-6-positive cells that ingest latex beads), 5–10% astrocytes (GFAP-positive cells), and 0–3% fibroblasts (vimentin-positive cells [44]). Additions of fluids from HIV-infected and control uninfected monocytes to the rat brain explants showed neuronotrophic activity. Numbers of NSE⁺ neurons treated with HIV-infected and control monocyte fluids for 5 d were two- to threefold higher than equal numbers of neurons treated with culture medium alone (Table 2 and Fig. 1). Fetal rat brain cells inoculated with fluids from HIV-infected or control uninfected U251 MG, U373 MG, or HTB 148 cells also showed no neuronotoxicity (Table 2).

Fluids from HIV-infected Monocyte–Glial Cell Interactions Produce Neuronotoxic Factors. The absence of neuronotoxic activity from HIV-infected monocytes or virus-infected neural cells led to assay of cell mixtures for neuronotoxic activities. Monocytes were infected with HIV-1_{ADA} for 7 d, harvested from teflon flasks, then placed onto equal numbers of neural cells. Fluids were harvested at 24 and 48 h after cocultivation then placed onto rat brain explant for assay of neuronotoxicity. In contrast to previous results, fluids from cocultures of HIV-infected monocytes with U251 MG, U373 MG, or HTB 148 astroglial cells were profoundly neuronotoxic (Table 2 and Fig. 1). Within 2 d after fluid addition neurons were swollen and vacuolated (Fig. 1). These fluids were also toxic for SK-N-MC neuroblastoma cells. The neural cell toxic activity was only produced in mixtures of HIV-infected monocytes and glia (U251 MG, U373 MG, and HTB 148 cell lines) (Table 2). Dose-response analysis of culture fluids from HIV-infected monocyte-astroglia (U251 MG) mixtures showed significant neuronotoxicity with dilutions of $\leq 1:20$ of the fluids. Indeed, a $\geq 50\%$ loss of viable neurons/well was evident when a 20-fold dilution of culture fluids of HIV-infected monocytes-astroglia was placed onto rat fetal neurons (Fig. 2). The toxic effects were cell specific. Fluids obtained from mixtures of HIV-infected monocytes and SK-N-MC (neuroblastoma)- or HIV-infected monocytes and endothelial cells showed no neuronotoxic activity (Table 2). The HIV-infected monocytes and U251 glial cells fluids were not toxic for rat or human astrocytes and fibroblasts (data not shown).

Mechanisms for Neuronotoxicity Analysis of Cytokine Gene Expression. A variety of cytokines may produce neurotoxicity and as such contribute to the pathogenesis of CNS disease. Two cytokines, IL-1 β and TNF- α , are associated with glial proliferation, neurotoxicity, and demyelination. Interestingly, these cellular effects are all prominent features of HIV-related encephalopathy. For example, TNF, at high concentrations, is a neurotoxin (28, 51, 52). Human astrocytes proliferate in response to TNF- α and IL-1 β (53, 54), and conditioned medium from LPS-treated astrocytes stimulates HIV-1 gene expression in monocytic cells (55). These observations

led us to investigate whether TNF- α and IL-1 β produced the neurotoxicity observed in supernatant fluids of HIV-infected monocytes and astroglia.

Initial experiments were performed to determine whether the addition of HIV-infected monocytes to astroglia resulted

Table 2. Neuron Survival and Differentiation in Rat Brain Explants Treated with Culture Fluids from Uninfected and HIV-1-infected Cells

Treated with:	Neuronal survival and differentiation
Tissue culture medium	
Medium alone	+
Medium with MCSF	+
Monocyte culture fluids	
Control monocytes	+ +
HIV-1 _{ADA} -infected monocytes	+ +
HIV and its products	
HIV-1 _{HTLVIII B}	+
HIV-1 ₂₄	+
HIV-1 _{ADA}	+
HIV gp120	+
HIV-infected monocytes cocultured with:	
Endothelial cells	+
SK-N-MC (neuroblastoma)	+
U251 MG (astroglia)	0
U373 MG (astroglia)	0
HTB 148 (neuroglia)	0
U251 MG astroglia cocultured with:	
Uninfected monocytes	+
HIV-1 _{ADA}	+
Freeze-thawed HIV-infected monocytes	+
Paraformaldehyde-fixed HIV-infected monocytes	+

Data show neuron survival and differentiation in fetal rat brain explants cultured for 10 d then treated for 5 d with monocytes media, conditioned media containing 20% (vol/vol) fluids from uninfected monocytes, HIV-1_{ADA} and HIV-1₂₄-infected monocytes, or with HIV-1_{HTLVIII B} or HIV-1_{ADA} virus stock ($>10^8$ HIV particles/ml by grid count on transmission electron microscopy), and 500 ng/ml recombinant HIV-1_{HTLVIII B} gp120. Conditioned media containing 20% (vol/vol) fluids from HIV-1_{ADA}-infected monocytes cocultured with endothelial cells, SK-N-MC (neuroblastoma), and U251 glial cells, and U251 MG astroglial cells incubated with HIV-1_{ADA}, freeze-thawed HIV-infected monocytes, paraformaldehyde-fixed HIV-infected monocytes, and uninfected monocytes were harvested after 24 h and assayed for neuronotoxicity. Neuron survival and extent of differentiation was estimated by cell morphology on phase contrast microscopy or on methanol-fixed, Wright-Giemsa-stained slides as outlined in Materials and Methods. Cell number was confirmed by MTT conversion as estimated by spectrophotometry at OD₄₉₀, and was scored as: 0, no neuritic outgrowth; +, dendritic outgrowth of 2–4 perikaryons distance in $\geq 50\%$ of cells/field; or + +, dendritic outgrowth of 4–8 perikaryons distance in $\geq 50\%$ of cells/field.

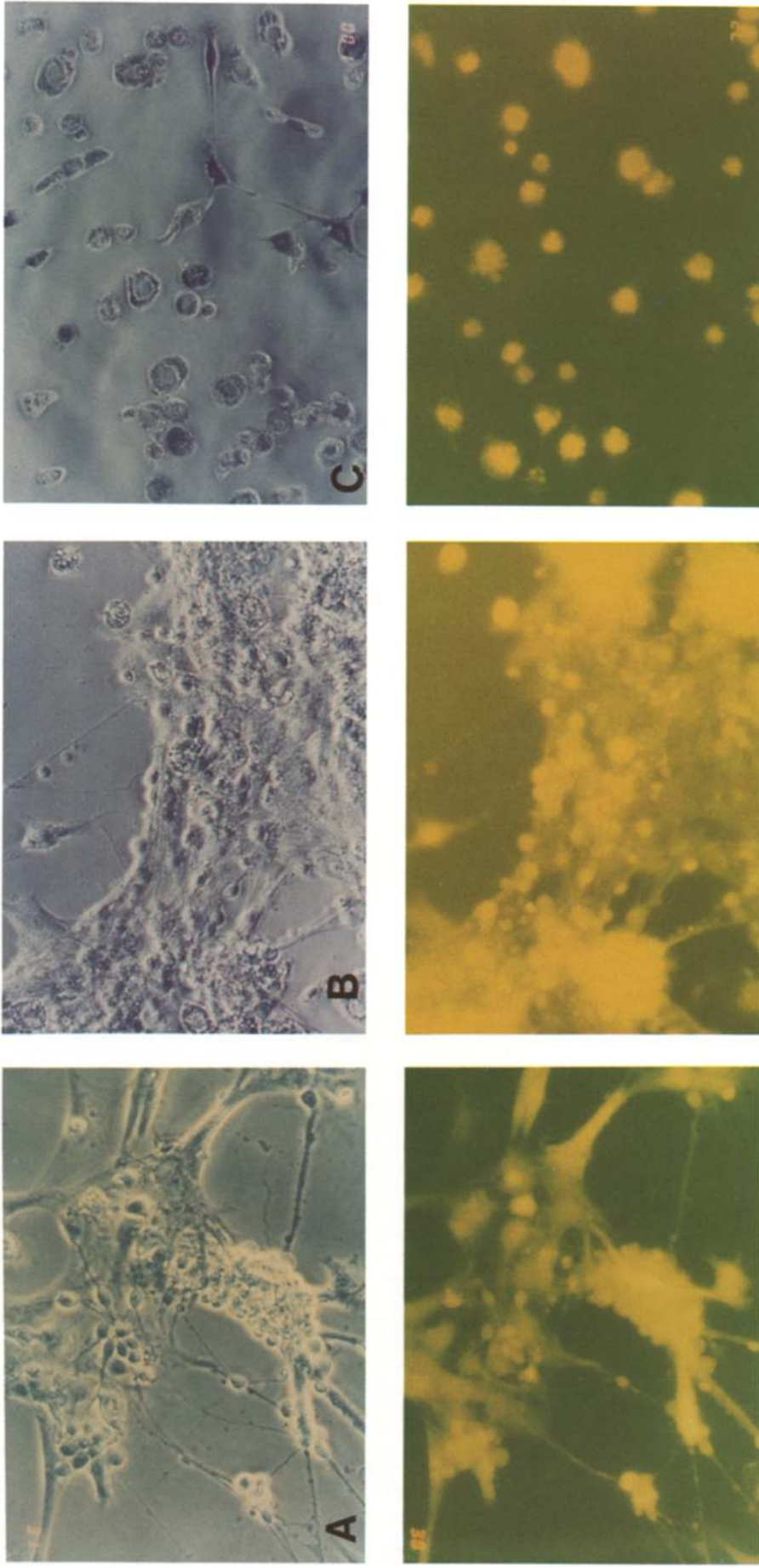


Figure 1. Rat neuronal cell cultures at 10 d were exposed to a 1:10 dilution of culture fluids from monocyte or monocyte-astroglial cocultures. Cultures were examined daily by phase-contrast microscopy. At day 4, cultures were stained for 90 min with calcein. The calcein dye is hydrolyzed into a yellow fluor by cytoplasmic esterases in living cells. (*top*) Phase microscopy; (*bottom*) identical fields under fluorescent illumination. (*A*) Neurons exposed to culture fluids of uninfected control monocytes show dense networks of finely branched dendrites and well-defined oval perikaria ($\times 30$). (*B*) Neurons exposed to fluids of HIV-infected monocytes are indistinguishable from control uninfected monocyte culture fluids ($\times 30$). (*C*) Neurons exposed to culture fluids of HIV-infected monocytes/astroglia (U251 MG) cocultures ($\times 30$). Neuronal cell bodies show extensive cytoplasmic vacuolization. Dendritic outgrowths are blunted or absent. Numerous glial cells are present throughout the field. This experiment is representative of three replicate experiments. Cocultivations were performed at a 1:1 cell ratio (HIV-infected monocytes/astroglia).

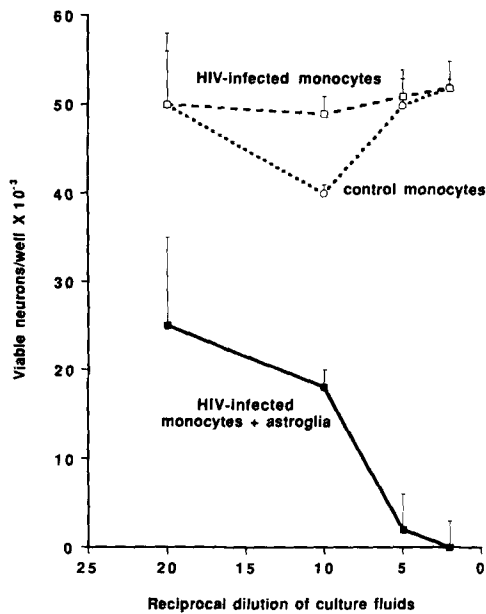


Figure 2. Correlation between neuronotoxicity and dilution of culture fluids from control uninfected monocytes, HIV-infected monocytes, and HIV-infected monocyte-astroglia (U251 MG cell) cocultures. Rat neuronal cell cultures at 10 d were exposed to twofold serial dilutions of culture fluids and analyzed for neuronotoxicity by counting neurons in triplicate wells in four microscope fields/well. Neuron survival was estimated by cell morphology on phase-contrast microscopy. Error bars represent mean \pm SD.

in cytokine gene expression. We examined the levels of TNF- α and IL-1 β by coupled RT-PCR in cell cocultures. The mRNA for the constitutive cellular enzyme, GAPDH, was examined as the reference cellular transcript. GAPDH mRNA amplification products were present at equivalent levels in all cell lysates. IFN- γ mRNA was not detected in any cell lysate (data not shown), demonstrating that a mixed T cell reaction was not an explanation for any cytokine mRNA expression observed. The mRNAs for IL-1 β , IL-1 α , TNF- α , and TNF- β in cell lysates of uninfected and HIV-infected monocyte cultures were absent (Fig. 3, lanes 1 and 2; and Table 3). However, the predicted 237- and 179-bp amplification products of TNF- α and IL-1 β mRNAs were readily seen in cell lysates of uninfected and HIV-infected monocytes and glia (U251 MG and HTB 148 cell lines) (Fig. 3, lanes 3 and 4, and 9 and 10). Interestingly, TNF- α and IL-1 β mRNAs were not detected in cell lysate and culture fluids of uninfected control or HIV-infected monocytes cocultured with endothelial cells (Fig. 3, lanes 7 and 8) or neuroblastoma cells (SK-N-MC cells) (Fig. 3, lanes 11 and 12). Control uninfected and HIV-infected U251 MG cells also showed no TNF- α and IL-1 β mRNAs (Fig. 3, lanes 5 and 6). Replicate experiments with primer pairs for three other cytokines (IL-1 α , TNF- β , or IL-6) (data not shown) were below the limits of PCR detection (Fig. 3). These results, taken together, show a selective and cell-specific induction of TNF- α and IL-1 β mRNA during the interaction between HIV-infected or control uninfected monocytes and glia.

The high levels of mRNAs were not always associated with

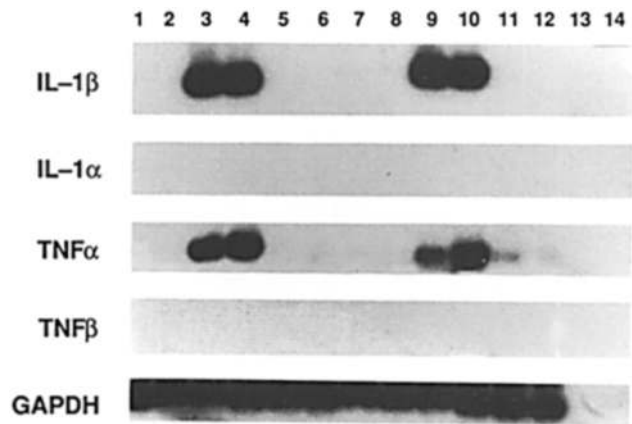


Figure 3. Induction of cytokine mRNA during HIV-infected monocyte-astroglial cell cocultivation. Monocytes cultured 7 d as adherent monolayers were exposed to HIV at an MOI of 0.01. 7 d after infection HIV-infected or control uninfected monocytes were cocultured 1:1 (monocytes/neural cells) with several different cell lines or primary cells for 24 h as outlined in Materials and Methods. RNA from cell lysates was extracted and mixed with antisense primers. After RT, cDNAs were amplified by PCR and the products of 25 cycles analyzed by Southern blot hybridization with cytokine-specific probes (see Table 1). Coupled RT-PCR amplification products from cell lysates of monocytes and/or neuronal cells cultured under different conditions are pictured in lanes: 1, uninfected monocytes; 2, HIV-infected monocytes; 3, uninfected monocytes cocultured with U251 glial cells; 4, HIV-infected monocytes cocultured with U251 glial cells; 5, uninfected U251 cell; 6, HIV-infected U251 cells; 7, uninfected monocytes cocultured with endothelial cells; 8, HIV-infected monocytes cocultured with endothelial cells; 9, uninfected monocytes cocultured with H4 HTB 148 cells; 10, HIV-infected monocytes cocultured with H4 HTB148 cells; 11, uninfected monocytes cocultured with SK-N-MC cells; 12, HIV-infected monocytes cocultured with SK-N-MC cells. Lane 13 is the λ plasmid control, and lane 14 is the PCR reaction run without RT. The latter specified that RNA not contaminating cellular DNA was amplified in the reaction.

similarly high levels of proteins (Table 3). TNF- α and IL-1 β protein and biological activity were seen only in coculture fluids of HIV-1-infected monocytes and glia (Table 3). The TNF- α and IL-1 β proteins were observed during coculture of HIV-infected monocytes and astroglial (U251 MG). Maximum levels were present 12–48 h after cocultivation. In a series of four replicate experiments maximum levels of TNF- α were 1,000–9,000 pg/ml (mean of 5,000), while IL-1 β levels ranged from 400 to 5,000 pg/ml (mean of 900) (Figs. 4 and 5). The results were confirmed by assays of TNF activity (Fig. 6). The underlying basis for this 10-fold difference was related to the levels of productive HIV infection. Peak cytokine levels occurred during the initial rise of RT activity, 3–5 d after virus infection, and diminished to baseline by day 10 (data not shown). TNF- α and IL-1 β proteins were also detected at low levels (<100 pg/ml) in cocultures of uninfected monocytes and astroglia (U251 MG and human astrocytes). The latter results suggest that the interactions seen between HIV-infected monocytes and glia are an extension of a normal physiological response. In all experiments assayed, the cytokine response mirrored the neuronotoxic response (data not shown).

Analysis of the Cytokine-producing Cell in Monocyte-Astroglia Cocultures. Cytokines produced during the interactions between HIV-infected monocytes and glia required viable mix-

Table 3. Levels of TNF- α mRNA and Protein after Coculture of HIV-infected Monocytes and the U251 Glial Cell Line

Cell treatments	TNF- α	
	mRNA	Protein pg/ml
Monocytes cultured with:		
Medium	0	0
LPS	++	2,250
Medium with MCSF	0	0
HIV-infected monocytes cultured with:		
Medium	0	0
Endothelial cell	0	0
SK-N-MC (neuroblastoma)	0	0
HTB 148	++	2,140
U251 glial cells	++	3,460
Cycloheximide then U251 MG cells	+++	1,300
Actinomycin D then U251 MG cells	+/-	90
U251 MG astroglial cells cultured with:		
Uninfected monocytes	++	50
HIV-1 _{ADA}	0	0
Freeze-thawed HIV-infected monocytes	0	0
Paraform.-fixed HIV-infected monocytes	0	0

TNF- α mRNA and protein in cell lysates and culture fluids of uninfected control, HIV-1_{ADA}-infected monocytes, and/or U251 MG astroglial cells after 24-h incubations with the treatments listed. Monocytes cultured 7 d as adherent monolayers were exposed to HIV at a MOI of 0.01. 1 wk after infection virus-infected monocytes were cocultured with media or equal numbers of: endothelial cells, SK-N-MC (neuroblastoma) cells, HTB 148 glial cells, U251 MG astroglial cells, cycloheximide then U251 MG astroglial cells, and cycloheximide then U251 astroglial cells. The U251 MG astroglial cells were incubated with: uninfected monocytes, HIV-1_{ADA}, mycoplasma-infected monocytes, and freeze-thawed or paraformaldehyde-fixed HIV-infected monocytes. Cytokine mRNA levels were detected by coupled RT-PCR amplifications from cell lysates. The extracted RNAs were mixed with antisense primers and, after RT, cDNA was amplified by PCR. The products of 25 cycles were analyzed by Southern blot hybridization. RNA was not detected (0), detected at low levels (+), was readily detected (++), or detected at high levels (+++). Cytokine levels in culture fluids were determined by ELISA.

tures of both cell types. When U251 MG or HTB 148 were mixed with 4% paraformaldehyde-fixed or freeze-thawed HIV-infected monocytes, cytokines were not detected (Tables 3). Pretreatment of HIV-infected monocytes with cycloheximide

or actinomycin D before coculture was used to determine if the infected monocytes were primary cytokine producers. Cycloheximide and actinomycin D were titrated to inhibit HIV mRNA and protein synthesis (positive controls for this assay system) and used at concentrations of 30 and 5 μ g/ml, respectively. In these assays, cycloheximide- or actinomycin D-treated monocytes were washed extensively before addition of U251 MG astroglial cells at equal cell concentrations. Analysis of TNF- α mRNA levels from cell lysates prepared from cycloheximide- or actinomycin D-treated cocultures showed increased and decreased levels of TNF- α mRNA, respectively (Fig. 7). However, both additions resulted in significant reductions in both TNF- α and IL-1 β proteins (Table 3; and unpublished observations). The higher levels of TNF- α protein seen after cycloheximide compared with actinomycin D treatment likely reflect the reversibility of cycloheximide after a 24-h wash out. That these inhibitors of RNA and protein synthesis had opposite effects on levels of TNF- α mRNA was not surprising. Indeed, previous reports show an upregulation of TNF- α mRNA in monocytes after both LPS and cycloheximide treatment (56, 57). These results coupled with the recent demonstration of TNF- α and IL-1 β protein in HIV-infected brain macrophages (58) support the notion that the macrophage is the primary cytokine producer. Furthermore, reproduction of these experimental findings with cocultures of HIV-infected monocytes and normal human fetal astrocytes (data not shown) lends support for the biological relevance of the described experimental system.

The role of TNF- α and IL-1 β in the observed neuronotoxicity was next explored. Toxicity of recombinant human (rh)TNF- α (Amgen Biologicals, Thousand Oaks, CA) and rhIL-1 β (Collaborative Research, Bedford, MA) was tested on rat fetal neuronal cultures as described above. Replicate experiments were performed with recombinant murine (rm)TNF- α (Genzyme, Boston, MA) and rmIL-1 β (Hoffman-La Roche, Inc., Nutley, NJ). The cytokine concentrations used in these assays were extrapolated from data from cocultures of HIV-infected monocytes and astroglia (1–10 ng/ml of recombinant protein). Inoculation of TNF- α and IL-1 β alone or in combination to fetal neuronal cultures produced no neuronotoxicity. Moreover, mycoplasma and endotoxin contaminations were ruled out as the neuronotoxin in the cell and viral preparations. Mycoplasma was not detected by hybridization assays in any of 10 randomly selected culture fluids. The levels of endotoxin contamination as detected by the *Limulus* amoebocyte lysate assay were <50 pg/ml. The addition of polymyxin B at 15 μ g/ml, a concentration known to inhibit the LPS-induced cytokine production, had no significant effect on cytokine levels observed in HIV-infected monocyte-astroglia cocultures. These results, taken together, demonstrate a clear association of cytokine and neuronotoxicity in cocultures of HIV-infected monocytes and astroglia.

Arachidonic Acid Metabolites Induced during Cocultures of HIV-infected Monocytes and Astroglia: Implications for Cytokine Regulation and Neuronotoxicity. Viable HIV-infected monocytes are required for the generation of cytokines and neuronotoxicity. Supernatant fluids from astroglial cells (U251 MG) mixed with 4% paraformaldehyde-fixed or freeze-thawed HIV-

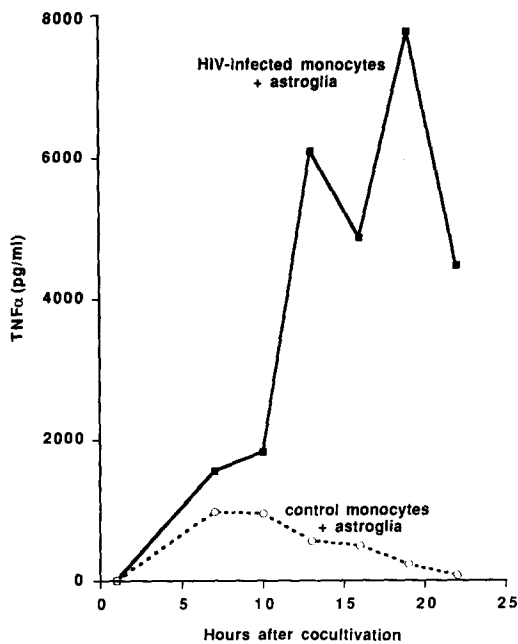


Figure 4. TNF- α levels in culture fluids of HIV-infected monocytes cocultured with astroglia (U251 MG cells). Adherent monocytes cultured for 7 d were exposed to HIV_{ADA} at a MOI of 0.01. At 7 d of infection HIV-infected and control uninfected monocytes were cocultured with equal numbers of astroglia. Aliquots of culture fluids were removed at various intervals and TNF levels measured by ELISA.

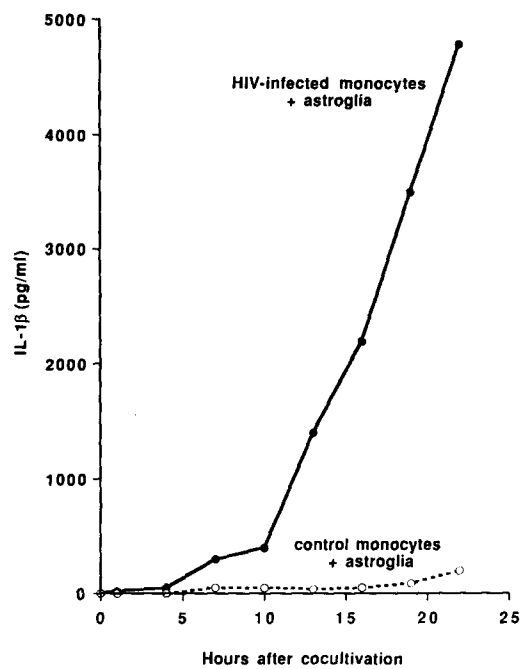


Figure 5. IL-1 β levels in culture fluids of HIV-infected monocytes cocultured with astroglia (U251 MG cells). Adherent monocytes cultured for 7 d were exposed to HIV_{ADA} at a MOI of 0.01. At 7 d of infection HIV-infected and control uninfected monocytes were cocultured with equal numbers of astroglia. Aliquots of culture fluids were removed at various intervals and IL-1 β levels measured by ELISA.

infected monocytes or HIV-1_{ADA} viral stock ($>10^8$ total particles/ml) each failed to produce neuronotoxicity. These results suggested yet another component for both cytokine and neuronotoxic responses. For several reasons, arachidonic acid metabolites were pursued as this possible missing component. First, arachidonic acid metabolites are upregulated in monocytes after incubation with the viral envelope glycoprotein, gp120 (50). Second, these metabolic products can regulate TNF- α and IL-1 β production in macrophages. Indeed, TNF causes amplification of arachidonic acid metabolites in response to IL-1, while PAF enhances TNF production (59–61). An autocrine/paracrine loop between arachidonic acid metabolites and cytokines and vice versa could explain the need for viable cell-to-cell interactions. Third, arachidonic acid metabolites play important roles in development neurobiology, neuronal function, and were reported as neuronotoxins (62–64). For these reasons, we determined whether arachidonate metabolites were produced during HIV-infected monocytes-astroglia interactions and whether they played a physiologically important role in this experimental system.

HPLC separation of arachidonic acid metabolic products released from uninfected control monocytes, HIV-infected monocytes, uninfected monocytes and U251 MG astroglial cells, and HIV-infected monocytes and U251 MG astroglial cells were evaluated. Cells were radiolabeled with [3 H]arachidonic acid for 18 h before coculture. The arachidonic metabolites were identified based on elution position standards (Table 4) and use of increasing polar solvents. The elution profiles of uninfected control (Fig. 8, *broken lines*) and HIV-

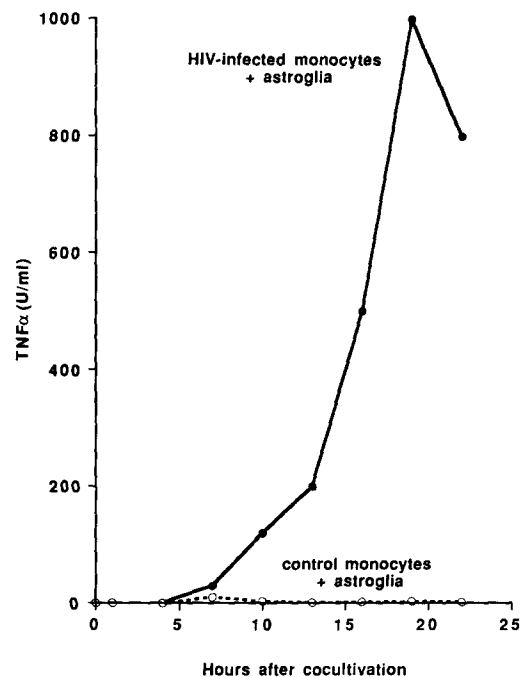


Figure 6. TNF activity in culture fluids of HIV-infected monocytes cocultured with astroglia (U251 MG cells). Adherent monocytes cultured for 7 d were exposed to HIV_{ADA} at a MOI of 0.01. At 7 d of infection HIV-infected and control uninfected monocytes were cocultured with equal numbers of astroglia. Aliquots of culture fluids were removed at various intervals, and TNF activity was measured by lysis of actinomycin D-treated L929 cells.

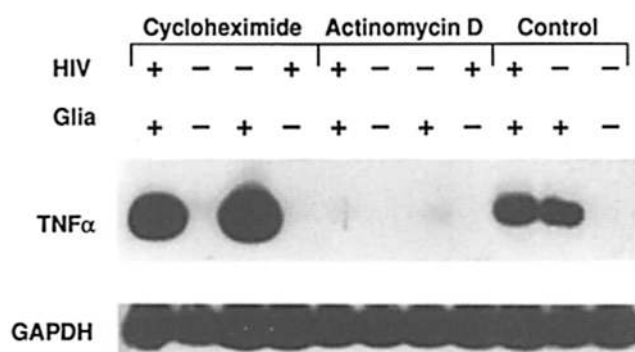


Figure 7. Induction of cytokine mRNA in HIV-infected monocyte and U251 MG cells after treatment with cycloheximide and actinomycin D. Monocytes cultured 7 d as adherent monolayers were exposed to HIV at an MOI of 0.01. 7 d after infection HIV-infected or control uninfected monocytes were treated with cycloheximide (30 μ g/ml) or actinomycin D (5 μ g/ml) for 1 h. After 3 h RNA was extracted from cell lysates and then mixed with antisense primers. After RT, cDNAs were amplified by PCR and the products of 25 cycles analyzed by Southern blot hybridization with a TNF- α -specific probe (see Table 1). Coupled RT-PCR amplification products from cell lysates of uninfected and HIV-infected monocytes cocultured with U251 MG astroglial cells and treated with or without cycloheximide, actinomycin D, or media (control) are shown.

infected monocytes (Fig. 8, *solid lines*) were virtually indistinguishable (Fig. 8, *b, d, and f*). Based on elution times of standard eicosanoids (Table 4), low levels of LTB₄, LTD₄, and 15-HETE were detected at 90 s (Fig. 8 *b*) but were not sustained at 90 and 180 min (Fig. 8, *d and f*). However, HPLC analysis of the arachidonate products released from HIV-infected monocytes after coculture with U251 MG astroglial cells (Fig. 8, *a, c, and e, solid line*) revealed signature profiles. Based on elution times of ³H standard (Table 4), the major products of the lipoxygenase pathway were LTB₄, LTD₄, and lipoxin A₄ (Fig. 8, *a, c, and e*). Although increased amounts of 5-HETE methyl ester and lactone were in HIV-infected cells the levels of these metabolites were indistinguishable between infected and uninfected (Fig. 8, *a, c, and e, block broken lines*) cell cocultures. Major increases were also seen in levels of 5-HETEs, methyl ester, and δ -lactone. Interestingly, LTB₄ levels were transient, produced at 90 s, but not sustained. This is in keeping with its known cyclical production (65). Moreover, cyclooxygenase products were identified only at low levels. Uninfected monocytes cocultured with astroglia produced 15-HETE, a metabolite found in low quantities in HIV-infected cells (Fig. 8, *a, c, and e*). The differences in the metabolic arachidonate profiles persisted through 90 min. At that time HIV-infected monocyte-astroglia cocultures showed a >20-fold increase in LTD₄ and uninfected cultures showed a >14-fold increase in 5-HPETE. LTB₄ oxidation products were cyclic but were always greater in HIV-infected cocultures. By 180 min, LTD₄ levels remained elevated in HIV-infected cocultures and increased greater than fourfold from cocultures of uninfected monocytes and astroglia.

The levels of PAF were evaluated by a quantitative RIA. Increased levels of PAF in cocultures of HIV-infected monocytes and astroglia were strongly associated with TNF- α

Table 4. Elution Time of Selected Eicosanoids

Metabolite	Time	Pathway
	<i>min</i>	
6-k-PGF ₁	6.0	Cyclooxygenase
LTB ₄ oxidation product	8.3	5-lipoxygenase
TXB ₂	11.2	Cyclooxygenase
PGF _{2α}	14.6	Cyclooxygenase
PGE ₂	18.5	Cyclooxygenase
Lipoxin A ₄	21.8	5, 15-lipoxygenase
LTC ₄	23.5	5-lipoxygenase
LTE ₄	26.4	5-lipoxygenase
LTB ₄	30.1	5-lipoxygenase
LTD ₄	41.0	5-lipoxygenase
15-HETE	44.0	15-lipoxygenase
15-HPETE	49.1	15-lipoxygenase
12-HETE	52.1	12-lipoxygenase
5-HETE	56.5	5-lipoxygenase
5-HPETE	60.4	5-lipoxygenase
15-HEDE	65.0	Internal standard
15-HETE methyl ester	71.5	15-lipoxygenase
5-HETE methyl ester	75.9	5-lipoxygenase
5-HETE δ lactone	79.5	5-lipoxygenase
Arachidonic acid	83.0	-
Eicosatrienoic acid	92.0	Internal standard

production (Table 5). To investigate a possible casual relationship between arachidonic acid metabolites and TNF- α , we added dexamethasone, an inhibitor of phospholipase A; indomethacin, a cyclooxygenase inhibitor; or NDGA, a lipoxygenase inhibitor (at concentration used), to cocultures of HIV-infected monocytes and astroglia, and measured TNF- α production (Table 6). Monolayers were infected with HIV at an MOI of 0.01 for 7 d before addition of U251 MG astroglial cells. The compounds were added together with equal numbers of astroglia. Both dexamethasone and NDGA markedly reduced the levels of TNF- α in supernatant fluids of these cocultured cells. Interestingly, indomethacin increased TNF- α levels. This likely reflected shunting of the arachidonate metabolites into the lipoxygenase pathway. The failure of NDGA to completely abrogate the TNF- α response suggests that PAF also participates in this cytokine response. Indeed, TNF- α is reproducibly detected after addition of PAF to cocultures of uninfected monocytes and astroglia (P. Genis, unpublished observations). Thus, arachidonic acid metabolites and PAF both likely participate in the TNF- α induction demonstrated in this experimental system.

Discussion

In the present study we demonstrated that the interactions between HIV-infected human monocytes and astroglia pro-

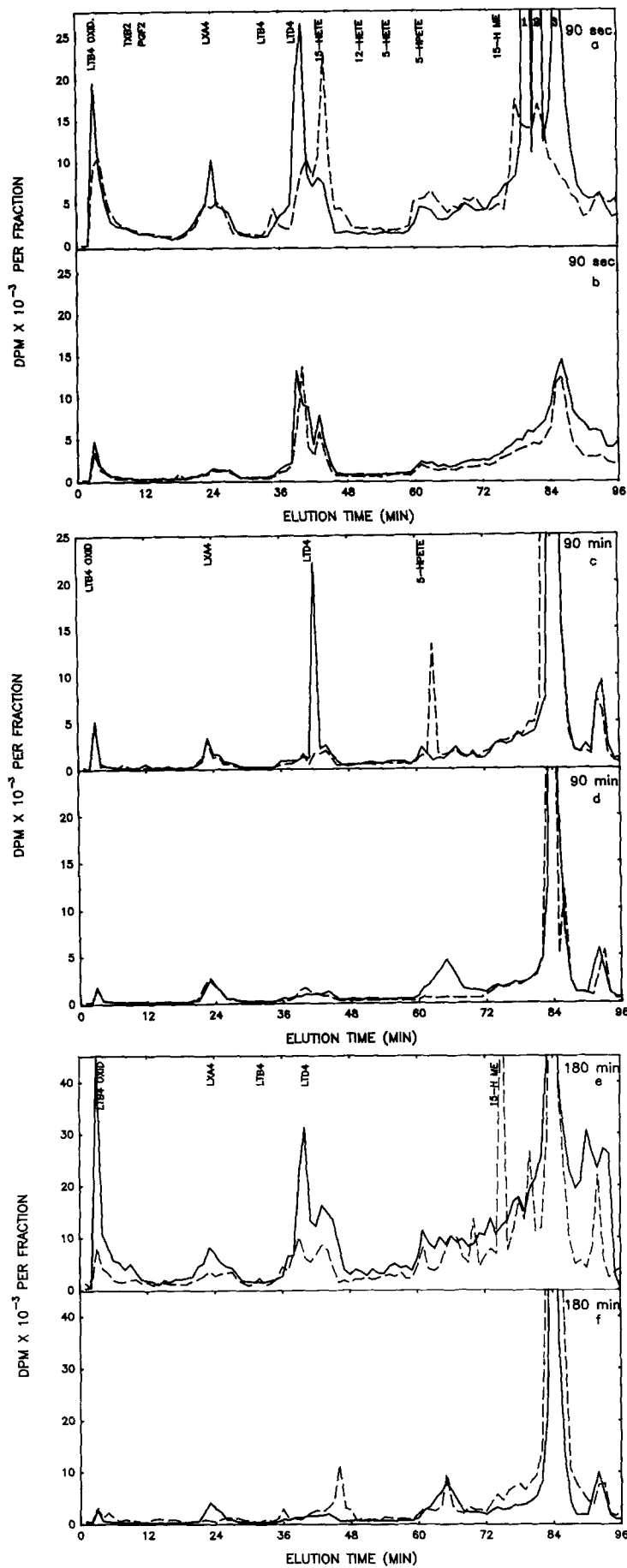


Figure 8. Arachidonic acid elution profiles of monocytes after HIV infection and cocultivation of HIV-infected monocytes and U251 astroglial cells. Adherent monocytes cultured for 7 d were exposed to HIV_{ADA} at a MOI of 0.01. At 7 d of infection HIV-infected and control uninfected monocytes (5×10^6 cells) were incubated and cocultured with equal numbers of astroglia (U251 MG cells). At various time intervals, 20 s to 180 min, the generation of arachidonic metabolites was examined. The metabolites were extracted, separated by HPLC, and analyzed quantitatively by determining DPM in the recovered fractions. The chromatograms were adjusted based on recovery of internal standards (see Table 6) as described in Materials and Methods. (a, c, and e) Cocultures of HIV-infected (solid lines) or uninfected control (broken line) monocytes and astroglial (U251 MG cells). (b, d, and f) HIV-infected (solid lines) and uninfected control (broken line) monocytes.

Table 5. PAF Levels after HIV Infection and Coculture of HIV-infected Monocytes and Astroglia

Cell cultures	PAF levels at:	
	60 min	120 min
	<i>pg/10⁶ cells</i>	
Monocytes cultured with:		
U251 MG astroglial cells	260	100
HIV-infected monocytes cultured with:		
Medium	360	100
U251 MG astroglial cells	990	670
U251 MG astroglial cells cultured with:		
Medium	130	200

PAF production in cultured cells. Monocytes cultured 7 d as adherent monolayers were exposed to HIV at a MOI of 0.01. 1 wk after infection virus-infected monocytes were cocultured with equal numbers of U251 MG astroglial cells. At 60 and 120 min of coculture, the cells were lysed and PAF levels determined by RIA.

duced high levels of IL-1 β and TNF- α . The IL-1 β and TNF- α responses were cytokine specific, correlated with neuronotoxicity, and occurred only during coculture of HIV-infected monocytes and astroglia. TNF- β , IL-1 α , IL-6, and IFN- γ were not produced. Mixtures of neuronal or endothelial cells and HIV-infected monocytes failed to elicit cytokines or neuronotoxins. Interestingly, the addition of TNF- α and IL-1 β alone or in combination to neurons at the concentrations found in HIV-infected monocyte-astroglia culture fluids did not produce neuronotoxicity. Viable glial cell-to-cell interactions were

Table 6. Effect of Arachidonic Acid Inhibitors on the TNF Levels after Coculture of HIV-infected Monocytes and Astroglia

Cell treatments	TNF- α protein at:	
	12 h	48 h
	<i>pg/ml</i>	
Medium	3,100	1,760
Dexamethasone (10^{-5} M)	50	30
Indomethacin (0.4 μ g/ml)	5,030	5,340
Nordihydroguaiaretic acid (NDGA) (5×10^{-5} M)	602	550

Effect of arachidonic acid inhibitors on TNF production. Monocytes cultured 7 d as adherent monolayers were exposed to HIV at an MOI of 0.01. 1 wk after infection virus-infected monocytes were cocultured with equal numbers of U251 MG astroglial cells. At the time of coculture the cells were incubated with medium, dexamethasone (10^{-5} M), indomethacin (0.4 μ g/ml) or NDGA (5×10^{-5} M). TNF production was measured by ELISA. Data represent means of duplicate determinations for one of three experiments performed.

required. Moreover, HIV-infected monocyte culture fluids, sucrose gradient-concentrated viral particles, and paraformaldehyde-fixed or freeze-thawed HIV-infected monocyte cell membranes failed to produce cytokine or neuronotoxic activity when placed on astrocytes. These data, taken together, suggested that other factor(s) were required for the observed responses. Two of these factors were identified as lipidic compounds derived from membrane phospholipids, including products of the 5-lipoxygenase pathway and PAF. These products, potent low molecular weight mediators of immune activation, were secreted within 90 s of the mixture between HIV-infected monocytes and astroglia. The large numbers of HIV-infected macrophages and astrogliosis in virus-infected brain tissue coupled with histological evidence of neuronotoxicity support the biological relevance of these observations.

Pathological outcomes of HIV-1 infection in brain tissue include neuronal loss, reactive astrogliosis, and myelin damage (66). Neuronal loss (67) is strongly associated with axonal and dendritic damage in the cortex and subcortex of affected individuals (68, 69). The paucity of productively infected neurons supports indirect mechanisms for neuronal damage seen during HIV disease (6–10). Indeed, viral antigens are found predominantly, if not exclusively, in cells of macrophage lineage (microglia, macrophages, and multinucleated giant cells).

It is tempting to attribute the pathogenesis of HIV encephalopathy to secretory products produced from HIV-infected macrophages or through the interactions between HIV-1-infected macrophages and astrocytes. Perhaps interplay, or "synergy," occurs between a number of toxic factors, including cytokines, arachidonic acid metabolites, and viral structural and/or regulatory proteins (gp120, tat, nef, etc.). Our results support such a notion and suggest that cytokines, viral proteins, and lipidic compounds produced during glial cellular interactions all play roles in the neuropathogenesis of HIV disease. Arachidonate metabolites and PAF may contribute to neuronal injury while stimulating TNF- α and IL-1 β production, resulting in astroglial proliferation (60, 61).

Macrophages play a pivotal role in the generation of the described neuronotoxin response. This result is not surprising. Indeed, macrophages can contribute to disease progression by several mechanisms. At sites of infection macrophages secrete scores of toxic effector molecules that damage tissue. Proliferation of autoreactive T cells through indigenous antigen presentation also leads to tissue damage and occurs in multiple sclerosis and rheumatoid arthritis (70, 71). In multiple sclerosis, cytokines produced from macrophages and T cells produce myelin damage. In rheumatoid arthritis, the secretion of monokines leads to alterations in endothelial cell adhesion and neutrophil influx. Ultimately, alterations in macrophage secretory and antigen-presenting functions leads to brain inflammation in multiple sclerosis and synovial hyperplasia of cartilage, tendons, and subchondral bone in rheumatoid arthritis.

The absence of critical regulatory signals between macrophages, microglia, and astrocytes may also contribute to the CNS disease process. Macrophages play important roles in

cell differentiation and tissue repair, and together with astrocytes regulate steady-state homeostatic CNS function. These functions provide an additional means for how HIV might disrupt neural function. For example, a critical homeostatic factor required for neuronal function might be lost because of HIV macrophage infection. However, the lack of supporting experimental evidence for this mechanism, the focality of tissue pathology and disease progression seen without large numbers of virus-infected cells, suggests that HIV-associated neuropathology occurs through the elaboration of toxic factors.

The astrocyte functions, in this experimental system, in a supportive role for cytokines and neuronotoxin induction. Although astrocytes can produce toxins (e.g., quinolinate) (72, 73) and secrete cytokines after appropriate stimulation, (e.g., IL-1, IL-3, TNF, and IL-6) (74), there is no evidence that the astrocyte produces any such factors *in vivo* (75). Moreover, cycloheximide and actinomycin D added to HIV-infected monocytes significantly reduced or eliminated cytokines within culture fluids of HIV-infected monocytes and astroglia. Nonetheless, astrocytes are required for cytokine and neurotoxin production. This suggests that they play an important role in the generation of CNS injury during HIV encephalopathy.

TNF and IL-1 likely contribute to the *in vivo* neuropathology of HIV infection. TNF regulates class I and II MHC antigens and induces proliferation of astrocytes. Additionally, TNF can cause myelin damage and lysis in oligodendrocytes and upregulates HIV gene expression in monocytic cells (51-54, 76). During HIV infection in brain, increased levels of TNF and/or IL-1 can induce intracellular adhesion molecule 1 expression (77) in endothelial cells and/or astrocytes. Thus, TNF can facilitate the production of inflammatory infiltrates in brain parenchyma and permit the penetration of virus-infected monocytes through the blood-brain barrier.

Several lines of evidence demonstrate that the regulation of TNF and IL-1 revolves around both the production of arachidonic acid metabolites and PAF. First, the addition of dexamethasone, a potent inhibitor of phospholipase A, to cocultures of HIV-infected monocytes and astroglia abrogates this cytokine response. Second, downregulation in TNF- α was

seen with NDGA, an inhibitor of the lipoxygenase pathway, while indomethacin, a cyclooxygenase inhibitor, increased TNF- α production. Third, a temporal relationship between production of LTB₄, LTD₄, lipoxin A₄, and PAF and TNF was shown. PAF enhances TNF production by inducing 5-lipoxygenase activity in macrophages (60, 61). PAF also causes the release of LTB₄ suggesting an interrelationship between the stimulation of TNF by PAF and endogenous lipoxygenase activity (61). TNF and IL-1 can also induce LTB₄ in human macrophages. The addition of PAF alone or in combination with LTB₄ into uninfected monocytes and astroglia cocultures induced TNF activity (P. Genis, unpublished observation). Last, the HIV glycoprotein (gp120) strongly upregulates (up to 40-fold) arachidonic acid metabolites and IL-1 activity in human monocytes (50). In this fashion HIV-1 gp120 may be inducing TNF- α and IL-1 β in primary brain cultures (78).

There is precedent for the ability of HIV-1-infected monocytes to trigger cytokine production from cells during cell-to-cell contact (e.g., IFN- α from PBMC and TGF- β from astrocytes) (30, 79). PBMC cocultured with HIV-infected monocytes release high levels of IFN- α activity. Levels of this IFN are associated with the interstitial pneumonitis seen in ungulate lentiviral infections and in children infected with HIV (80). The release of TGF- β , a potent chemotactic factor, is initiated from the interactions between HIV-infected macrophages and astrocytes. The production of TGF- β in brain likely permits recruitment of HIV-infected monocytes into brain, providing a mechanism for efficient viral spread and disease (79). The exact secretory factors that regulate neural injury in HIV disease likely revolve around combinations of factors including arachidonic acid metabolites, cytokines, and other toxins. Nonetheless, the mechanism of cytokine and neuronotoxic factor induction described in this report coupled with identifiable compounds that augment the neurological response should prove helpful in understanding the basic mechanisms underlying HIV-induced CNS injury and for providing therapeutic strategies for AIDS-associated encephalopathy.

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