Regulation of Nitric Oxide Synthase Activity in Human Immunodeficiency Virus Type 1 (HIV-1)–infected Monocytes: Implications for HIV-associated Neurological Disease

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Summary

Mononuclear phagocytes (monocytes, macrophages, and dendritic cells) play major roles in human immunodeficiency virus (HIV) persistence and disease pathogenesis. Macrophage antigen presentation and effector cell functions are impaired by HIV-1 infection. Abnormalities of macrophage effector cell function in bone marrow, lung, and brain likely result as a direct consequence of cellular activation and HIV replication. To further elucidate the extent of macrophage dysfunction in HIV-1 disease, a critical activation-specific regulatory molecule, nitric oxide ($NO^{\cdot}$), which may contribute to diverse pathology, was studied. Little, if any, $NO^{\cdot}$ is produced by uninfected human monocytes. In contrast, infection with HIV-1 increases $NO^{\cdot}$ production to modest, but significant levels (2–5 μM). Monocyte activation (with lipopolysaccharide, tumor necrosis factor α, or through interactions with astroglial cells) further enhances $NO^{\cdot}$ production in HIV-infected cells, whereas its levels are diminished by interleukin 4. These results suggest a possible role for $NO^{\cdot}$ in HIV-associated pathology where virus-infected macrophages are found. In support of this hypothesis, RNA encoding the inducible NO synthase (iNOS) was detected in postmortem brain tissue from one pediatric AIDS patient with advanced HIV encephalitis. Corresponding iNOS mRNA was not detected in brain tissue from five AIDS patients who died with less significant brain disease. These results demonstrate that HIV-1 can influence the expression of NOS in both cultured human monocytes and brain tissue. This newly described feature of HIV–macrophage interactions suggests previously unappreciated mechanisms of tissue pathology that result from productive viral replication.

During the course of HIV infection, many patients develop neurocognitive defects, a condition termed the HIV-1–associated cognitive/motor complex (1, 2). The AIDS dementia complex (ADC), a severe form of this impairment, occurs in 20–30% of immunosuppressed patients with neurological deficits (1, 2). Although the mechanisms of HIV-1–induced central nervous system (CNS) disorders remain unknown, they are associated with productive viral replication, particularly in brain macrophages, multinucleated giant cells, and microglia (3, 4). Our recent work demonstrated that interactions between HIV-infected monocytes and astroglial cells produce high levels of proinflammatory cytokines (TNF-α, IL-1β), platelet-activating factor (PAF) (5), and eicosanoids (6). These potent, cell-derived effector molecules are cytotoxic when added to primary neuronal cultures and are also detected in the cerebrospinal fluid of HIV-infected subjects with neurological deficits (7–9).

The final common pathway for neuronal injury in HIV-1 disease likely revolves around stimulation of neuronal *N*-meth-

[1] The first two authors contributed equally to the paper.

yl-D-aspartate (NMDA) receptors (10–13). Glutamate neurotoxicity in primary neuronal cultures induced by stimulation of NMDA receptors is mediated in part by nitric oxide ($NO^{\cdot}$)[2] (14–17). $NO^{\cdot}$ is a powerful endogenous mediator for numerous physiological responses that are implicated in the antimicrobial, antiviral, and antiproliferative activities (18–20), as well as in many manifestations of tissue and, in particular, of brain injury (19, 21–23).

Production of $NO^{\cdot}$ is regulated in seemingly complex ways that are host dependent. Treatment of murine macrophages with IFN-γ and LPS induces the expression of iNOS, a gene encoding an inducible isoform of NOS that produces large amounts of $NO^{\cdot}$ from a guanidino nitrogen of L-arginine (19, 20). The induction of iNOS activity in human monocytes has been variably reproducible (24–29). The underlying cause for this species heterogeneity has not been resolved, but may underscore important intracellular signaling differences between mouse and human macrophages, as well as variations in cultivation conditions of cells. In this paper we demonstrate induction of iNOS in HIV-infected human monocytes, a population of cells that has been used as model of tissue macrophage function (30, 31). We also show that this NOS induction is subject to both positive and negative regulation by the immune system cytokine network. HIV-1 can both prime monocytes for $NO^{\cdot}$ induction by LPS or TNF-α, as well as induce $NO^{\cdot}$ de novo. Although the levels of $NO^{\cdot}$ produced by HIV-infected human monocytes are not striking, they are significant across donor and assay systems, and may participate in producing the CNS injuries associated with HIV-1 infection of brain macrophages.

Materials and Methods

Isolation and Propagation of Human Monocytes and Astroglia. 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 the criteria of cell morphology on Wright-stained cytosmears, by granular peroxidase, and by nonspecific esterase. Monocytes were cultured as adherent monolayers (10^6 cells/ml) in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% pooled human sera, 50 μg/ml gentamicin, and 1,000 U/ml highly purified (<0.01 ng/ml endotoxin) recombinant human M-CSF (Genetics Institute, Cambridge, MA) for 7 d.

The U251 MG human astroglial tumor-derived cell line was obtained from D. Bigner (Duke University, Durham, NC) (32). The cells were grown as adherent monolayers in DMEM with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) (or human serum in co-culture experiments) and 50 μg/ml gentamicin.

HIV Infection of Monocytes. Adherent monocytes cultured for 7 d were exposed to 10^5 $TCID_{50}$/ml of a monocytotropic viral strain, $HIV\text{-}1_{ADA}$. 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 as described previously (6).

Coupled RT-PCR analysis of RNA. RNA was isolated from 2–5 × 10^6 cells by acid guanidium thiocyanate-phenol-chloroform extraction using RNAzol (Biotecx Laboratories, Inc., Houston, TX) and reverse transcribed by Moloney murine leukemia virus RT using random hexamers as primers (all reagents were included in the GeneAmp RNA PCR Kit; Perkin-Elmer Corp., Norwalk, CT). Complementary DNA was divided into several aliquots that were amplified with different primer pairs. To increase specificity of the analysis, products of the PCR were visualized in some cases by Southern hybridization, using oligonucleotides corresponding in sequence to an internal portion of the predicted fragment as the probe. PCR primers used for NOS RNA detection were designed to allow discrimination between the constitutive (cNOS) and inducible (iNOS) forms of NOS by exploiting the fact that the cDNA for both mouse and human iNOS has a gap of about 130 bp 5′ to the flavinmononucleotide (FMN) binding site, compared to the constitutive form (33–35). Therefore, amplification of iNOS with these primers produces a band of 614 bp, whereas constitutive NOS RNA would give rise to a product of 748 bp. Only the 614-bp product was detected in monocytes and in the brain samples; therefore it is designated iNOS. Control experiments with primers that specifically amplify a 3′ untranslated region of iNOS mRNA (Clontech, Palo Alto, CA) produced identical results and demonstrated the validity of the RT-PCR assay. Sequences of the primers and probes used in this study are shown in Table 1. PCR reactions were assembled according to the protocol supplied with the kit. Amplification was performed either for 28 cycles (30s, 94°C; 30 s, 50°C; 1 min, 72°C) for HIV *pol*, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers; or for 40 cycles (45 s, 94°C; 45 s, 58°C, 2 min, 72°C) with NOS primers. Amplifications were preceded by denaturation step (3 min, 94°C) and followed by a final extension period (5 min, 72°C).

Analysis of $NO^{\cdot}$ in Culture Fluids. $NO^{\cdot}$ production was initially assessed by total nitrite formation. Nitrites were measured by the Griess reaction (36). 100 μl of culture medium without phenol red was mixed with 100 μl of Griess reagent (1% sulfanilic acid, 0.1% D-naphthyl ethylenediamine [both from Sigma Chemical Co.] in 5% phosphoric acid) in a microplate, and results were read after a 20-min incubation at room temperature in an ELISA reader (Bio-Tek Instruments, Burlington, VT) at 570 nm. Standard curves of various fixed concentrations of sodium nitrite (Sigma Chemical Co.) were generated alongside the experimental samples.

Electron Paramagnetic Resonance Detection of Free $NO^{\cdot}$ by Spin-Trap Methods. A useful technique for detecting the presence of $NO^{\cdot}$ is the use of the Fe-diethyldithiocarbamate $(DETC)_2$ complex to spin-trap $NO^{\cdot}$ in the form of the ternary paramagnetic complex $NO\text{-}Fe\text{-}(DETC)_2$. The advantage of this method is that this $NO^{\cdot}$-specific spin-trap can be detected in the liquid or solid aqueous phase (37–39). To perform this sensitive spin-trap method, cell cultures were prepared as usual with HIV-infected or uninfected macrophages (which served as the control). After 5–7 d in culture, DETC (2.5 mg/ml) was added to the culture fluid, followed by the combination of Fe^{2+} citrate (0.25 mg/ml), and sodium citrate (1.25 mg/ml). After 30 min, all samples (HIV-infected and controls) were treated with 95% ethanol to a final concentration of 70% by volume to inactivate the virus. The fluid was then transferred to a 4-mm diameter quartz glass electron paramagnetic resonance (EPR) spin tube and freeze-quenched in liquid nitrogen. Samples were sent on dry ice by express courier to the EPR facility in the Department of Chemistry at Harvard University. They were kept in liquid nitrogen until measurement. The EPR spectra were monitored in

[2] *Abbreviations used in this paper:* DETC, diethyldithiocarbamate; EPR, electron paramagnetic resonance; gp, glycoprotein; iNOS, inducible nitric oxide synthase; NMA, N^G-methyl-L-arginine; $NO^{\cdot}$, nitric oxide; RT, reverse transcriptase.

Table 1. *Nucleotide Primers in Coupled RT-PCR Assay*

Amplification product size	Nucleotide position	Primer	Sequence
iNOS (35)	1484-1503	Sense	CCAGAAGCAGAATGTGACCA
614 bp	2098-2076	Antisense	TACATGCTGGAGCCAAGGCCAAA
	1563-1589	Probe	GCCAGGGGGGGCTGCCCTGCAG ACTGG
β-actin (58)	3076-3096	Sense	GACTTAGTTGCGTTACACCC
349 bp	3425-3405	Antisense	CCTCCCCTGTGTGGACTTGG
HIV *pol* (59)	2317-2334	Sense	GAAGCTCTATTAGATACAGG
282 bp	2595-2576	Antisense	TCCTGGCTTTAATTTTACTGG
	2399-2420	Probe	GGAATTGGAGGTTTATCAAAGT
GAPDH (60)	199-217	Sense	CCATGGAGAAGGCTGGGG
195 bp	394-374	Antisense	CAAAGTTGTCATGGATGACC
	280-299	Probe	CTAAGCAGTTGGTGGTGCA

a liquid nitrogen flow-through system at a temperature of −100°K on a ESP 300 E instrument (Brüker, Billerica, MA).

Brain Autopsy Materials. Cases 1–5 were HIV-1 infected, whereas cases 6–8 were control (uninfected). Case 1 was a 37-yr-old male with clinical and pathologic evidence of AIDS dementia complex; case 2 was a 35-yr-old male with chronic panencephalitis secondary to HIV-1 infection; case 3 was a 6-mo-old male with severe HIV-1 encephalitis with numerous multinucleated giant cells and inflammatory cell infiltrates in the brain; case 4 was a 12-yr-old male with mild HIV-1 encephalitis; case 5 was a 42-yr-old male with mild HIV-1 encephalitis; case 6 was a 61-yr-old female diagnosed with multiple sclerosis; case 7 was a 71-yr-old male with squamous cell carcinoma of the lung but no pathological abnormalities in his brain tissue; and case 8 was a 51-yr-old male with chronic active hepatitis who died of a hepatocellular carcinoma and liver failure.

Results

HIV-1–infected Monocytes Produce NO·. Cells were cultured for 7 d in the presence of M-CSF before viral infection. At day 6 after HIV-1_{ADA} infection, modest but significant levels of nitrites could be detected in the culture medium (Fig. 1 *A*). Peak nitrite production occurred 1 d after the peak of RT activity (Fig. 1 *A*). RT-PCR analysis revealed induction of iNOS RNA by day 6 after infection, but iNOS mRNA was never detected in uninfected control cultures (Fig. 1 *A*, *inset*). Production of nitrites by HIV-infected monocytes correlated with the level of virus replication. We observed that significant levels of nitrites (above the background of ~0.9 μM) were detected when RT activity reached ≥0.5 × 10^6 cpm/ml. However, cells from two out of six donors did not produce detectable NO· even after high levels of viral replication were established. These cells also did not produce NO· after stimulation with either LPS or TNF-α. To detect the presence of authentic NO·, we used a spin-trapping technique that is sensitive and specific for the free radical (38). EPR detection of this spin–trap complex demonstrated the presence of authentic NO· in culture fluids of HIV-infected macrophages as early as 5 d after infection (Fig. 1 *B*). NO· in culture fluids of uninfected (control) cells was undetectable using this detection system.

A recent report (25) suggested that treatment of monocytes with HIV-1 glycoprotein (gp)120 induces production of NO·. In our system, addition of purified gp120 at 4 or 40 μg/ml induced low levels of nitrites (Fig. 2). However, this effect was much smaller than the production of NO· by HIV-infected cultures. Cross-linking of the macrophage CD4 receptor with anti-CD4 antibody or addition of equal (by p24) amounts of heat-inactivated HIV-1 also failed to induce significant levels of nitrite production (data not shown).

HIV-1 Infection Can both Prime and Induce NO· Production by Human Monocytes. Since LPS is known to induce NOS in mouse macrophages (40), we tested it along with the proinflammatory cytokine TNF-α for the ability to induce NO·-related effects. LPS and TNF-α were added to HIV-infected monocytes 4 d after infection when neither NO· nor NOS mRNA was detectable (Fig. 1 *A*). As shown in Fig. 3, *A* and *B*, both agents induced production of NO· by 24 h after their addition. Production of NO· was partially blocked by N^G-methyl-L-arginine (NMA) (Fig. 3, *A* and *B*) and was completely inhibited in arginine-deficient medium (Fig. 3 *A*). In agreement with previously reported results (41), TNF-α and LPS did not significantly alter virus replication in our system, likely because the observation periods were of limited duration. Therefore, the effect of TNF-α and LPS on iNOS appears not dependent on the alteration of viral replication, but rather on the triggering of an already primed cell. This effect of LPS was dose dependent, with maximal induction achieved at ~10 ng/ml (Fig. 3 *B*). The antiinflammatory cytokine IL-4 at 5 ng/ml diminished the effect of LPS stimulation (Fig. 3 *B*), demonstrating that the NO·-mediated re-

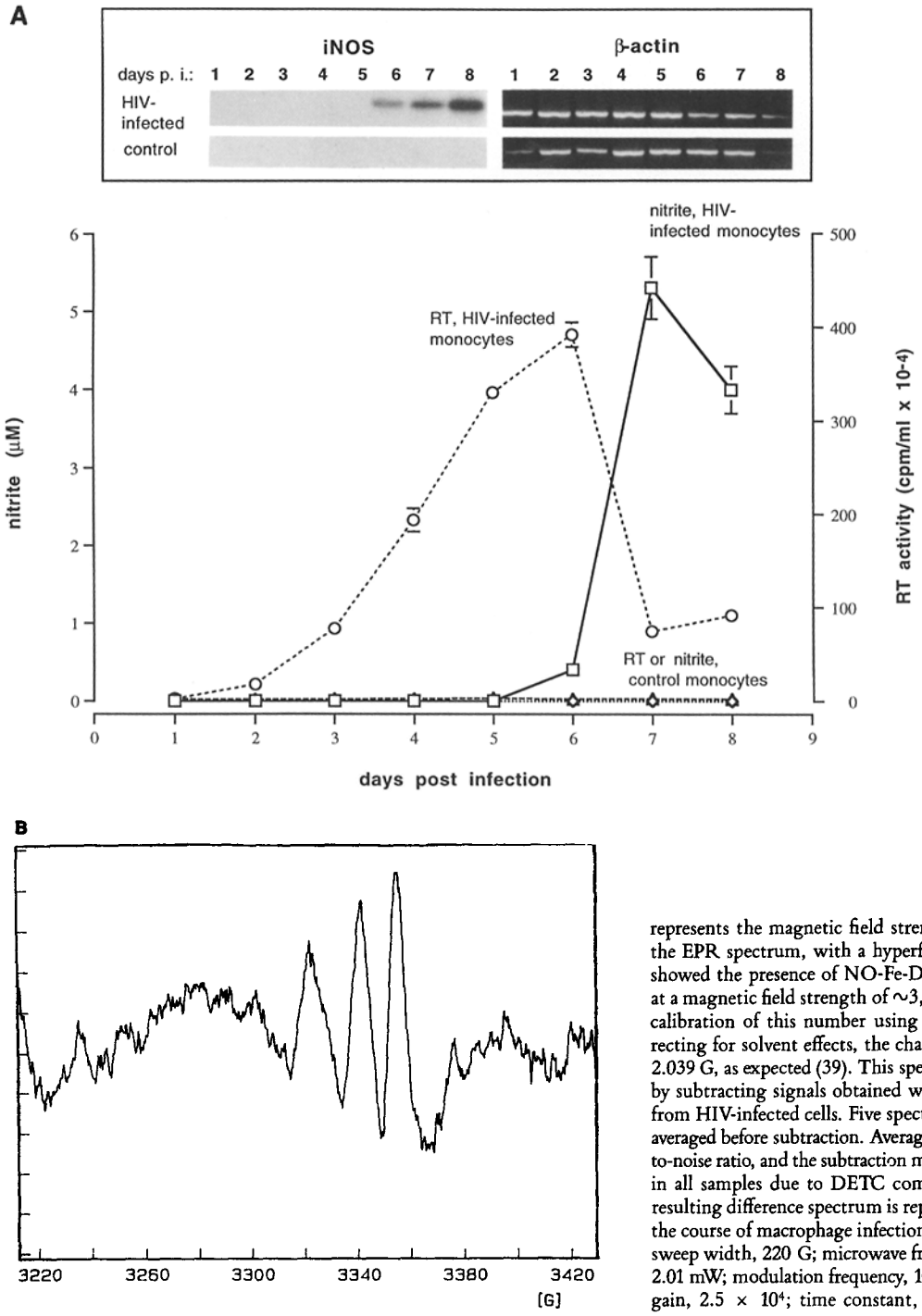

Figure 1. Analysis of NOS activity and iNOS mRNA in cultured human monocytes. (*A*) RT activity and nitrite concentration were determined in culture media at daily intervals in HIV-infected or control (uninfected) monocyte cultures in the absence of additional stimulation. Values obtained for control cells (0.9 μM) were subtracted from all corresponding values. Each sample was assayed in triplicate and the results are the mean ± SD. Results of the RT-PCR analysis of HIV-infected and uninfected (*control*) cultures are presented in the inset. Days after infection are shown above the lanes. PCR was performed with either iNOS-specific primers (PCR product was visualized by Southern hybridization with a ^{32}P-labeled NOS-specific probe), or with β-actin–specific primers (PCR product was visualized by ethidium bromide staining). Four experiments with cells from different donors were performed and results of a representative experiment are shown. (*B*) EPR illustration of NO· production by HIV-infected monocytes 5 d after infection. The ordinate axis is in arbitrary units representing the strength of the spin–trap signal detecting NO·, and the abscissa represents the magnetic field strength. A characteristic triplet signal on the EPR spectrum, with a hyperfine coupling constant of 13 gauss (*G*), showed the presence of NO-Fe-DETC (39). The triplet peak was found at a magnetic field strength of ~3,340 G, as illustrated in the figure. After calibration of this number using the frequency of stimulation and correcting for solvent effects, the characteristic triplet signal was centered at 2.039 G, as expected (39). This spectrum represents the difference obtained by subtracting signals obtained with samples of uninfected macrophages from HIV-infected cells. Five spectra were acquired from each sample and averaged before subtraction. Averaging was performed to increase the signal-to-noise ratio, and the subtraction method was used to remove signals present in all samples due to DETC complexing with endogenous Cu^{2+}. The resulting difference spectrum is representative of six such samples run over the course of macrophage infection. EPR parameters: center scan, 3318 G; sweep width, 220 G; microwave frequency, 9.405 GHz; microwave power, 2.01 mW; modulation frequency, 100 kHz; modulation amplitude, 7.08 G; gain, 2.5 × 10^4; time constant, 524.88 s.

sponses can be regulated both positively (e.g., by TNF-α) and negatively (e.g., by IL-4) by the immune system.

Whereas LPS-mediated boost of nitrite production by HIV-infected monocytes suggests a priming effect of HIV-1 infection, results presented in Fig. 1 *A* indicate that HIV-1 infection per se can induce production of NO·, although at later time points. To elucidate this question, we added LPS to monocyte cultures at various intervals after infection and measured NO· production by LPS-stimulated and unstimulated HIV-infected monocytes. For this experiment, cells were grown in bulk cultures in Teflon flasks. This procedure allowed us to avoid well-to-well variations associated with cultures grown in multi-well plates, and also produced a more rapid kinetics of NO· production in unstimulated cells (Table 2). Results presented in Table 2 indicate that the LPS-mediated boost of nitrite production can be seen only at day 5, when virus replication is relatively low. At later time points, stimulation with LPS did not significantly affect production of NO·.

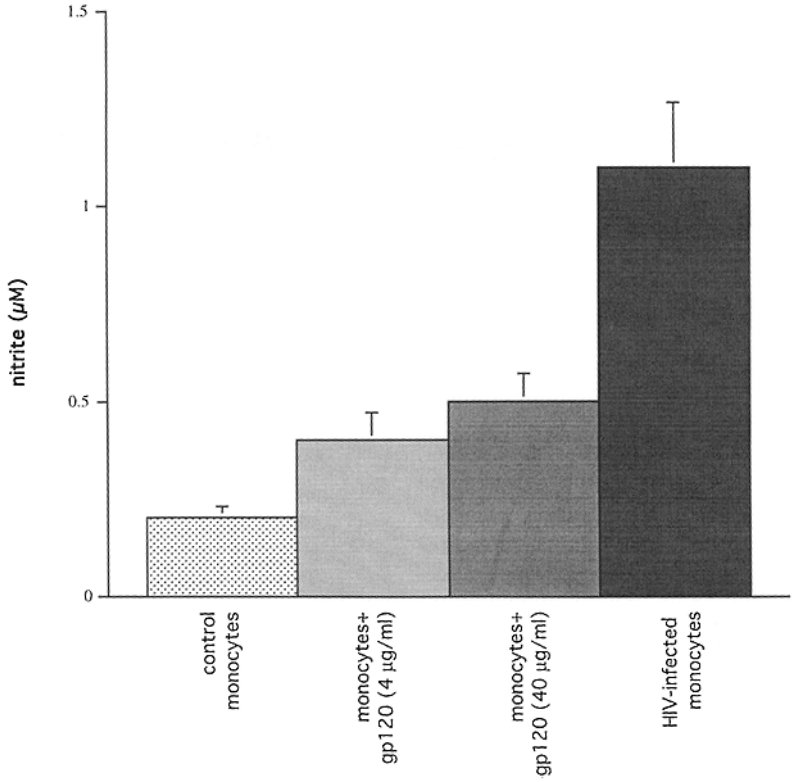

Figure 2. Nitrite production by human monocytes incubated with gp120. Purified human monocytes were cultured 7 d before infection with HIV-1_{ADA}. For uninfected monocytes, cultivation was continued for an additional 5 d, then medium was changed to a new one containing recombinant gp120 from HIV-1_{SF2} (obtained from NIH AIDS Research and Reference Reagent Program, Cat. #386) and cells were incubated for an additional 24 h before analysis of nitrites. HIV-infected cells were cultured for 5 d; then the medium was changed and nitrite production was measured by the Griess reaction after 24 h of incubation. Two experiments with cells from different donors were performed, and results of a representative experiment are shown. For each time point, three separate wells were prepared and analyzed, and the presented results are the mean ± SD.

NO· Is Produced in Co-cultures of HIV-infected Monocytes with Astroglia. As a first step in determining the role of NO· in HIV-induced CNS disease, we studied NO· regulation in an experimental in vitro cell culture model. This system includes the major cell types (macrophages and astroglia) thought to mediate HIV-induced neuronal injury (5, 6). 7 d after plating and 4 d after viral inoculation, equal numbers of U251 astroglial cells were added to the virus-infected monocytes. A significant increase in nitrite concentration in the culture medium occurred 48 h after the initiation of co-culture of the HIV-infected monocytes with astroglial cells (Fig. 4). Increased levels of nitrites were also detected in co-cultures of uninfected monocytes and astroglial cells (Fig. 4). This latter result suggested that HIV infection amplified a possibly normal set of physiological responses between monocytes and astroglia. Similarly, TNF-α activity was detectable in media from co-cultures of U251 astroglial cells and uninfected monocytes, but not in cultures of uninfected monocytes alone. This astrocyte-dependent appearance of bioactive TNF was further enhanced if the monocytes were infected with HIV-1 (Fig. 5). Since TNF-α induces NOS in human astrocytes (42, 43) and HIV-infected macrophages (Fig. 3 *A*), but not in normal macrophages (Fig. 3 *A*), NOS in uninfected co-cultures may well reflect an astrological cell source.

iNOS RNA in the Brain Tissue of a Pediatric Patient with Severe HIV Encephalitis. The relevance of the observed phenomena to the pathogenesis of HIV encephalitis was assessed by RNA PCR analysis of brain tissue samples obtained at autopsy from HIV-infected subjects. Samples were obtained from frontal lobe and included both white and gray matter. Results of PCR analysis of brain samples with HIV- and iNOS-specific primers are shown in Fig. 6. A product of 614 bp, characteristic of iNOS was amplified from cDNA prepared from brain tissue of a pediatric patient with severe HIV encephalitis (patient 3). This patient presented the characteristic features of advanced HIV-related brain disease, with widespread inflammation and multinucleated giant cells. We did not detect iNOS RNA in samples from other patients with lesser degrees of HIV encephalitis, or from controls without microscopic evidence of neuropathology (Fig. 6).

Discussion

This study demonstrates that HIV-1 infection of human monocytes results in the appearance of the inducible isoform of NOS and is accompanied by modest but significant production of NO·. The effect of HIV-1 infection is likely not strain specific, since we observed the same phenomenon with HIV-1_{SF162} (data not shown) and HIV-1_{ADA} strains. This induction of NO· synthesis occurs late during viral infection. However, activation of HIV-infected monocytes by LPS or TNF-α results in NO· production at earlier time points, before any NO· can be detected in HIV-infected but otherwise unstimulated cells. Later in infection, NO· production declines and cannot be boosted by LPS stimulation, probably because monocytes are already fully activated for NO· production. This suggests that HIV-1 can both prime and induce human monocytes for expression of iNOS and synthesis of NO·.

NMA, a competitive inhibitor of NOS, inhibited production of NO· by HIV-infected human monocytes at IC_{50} of

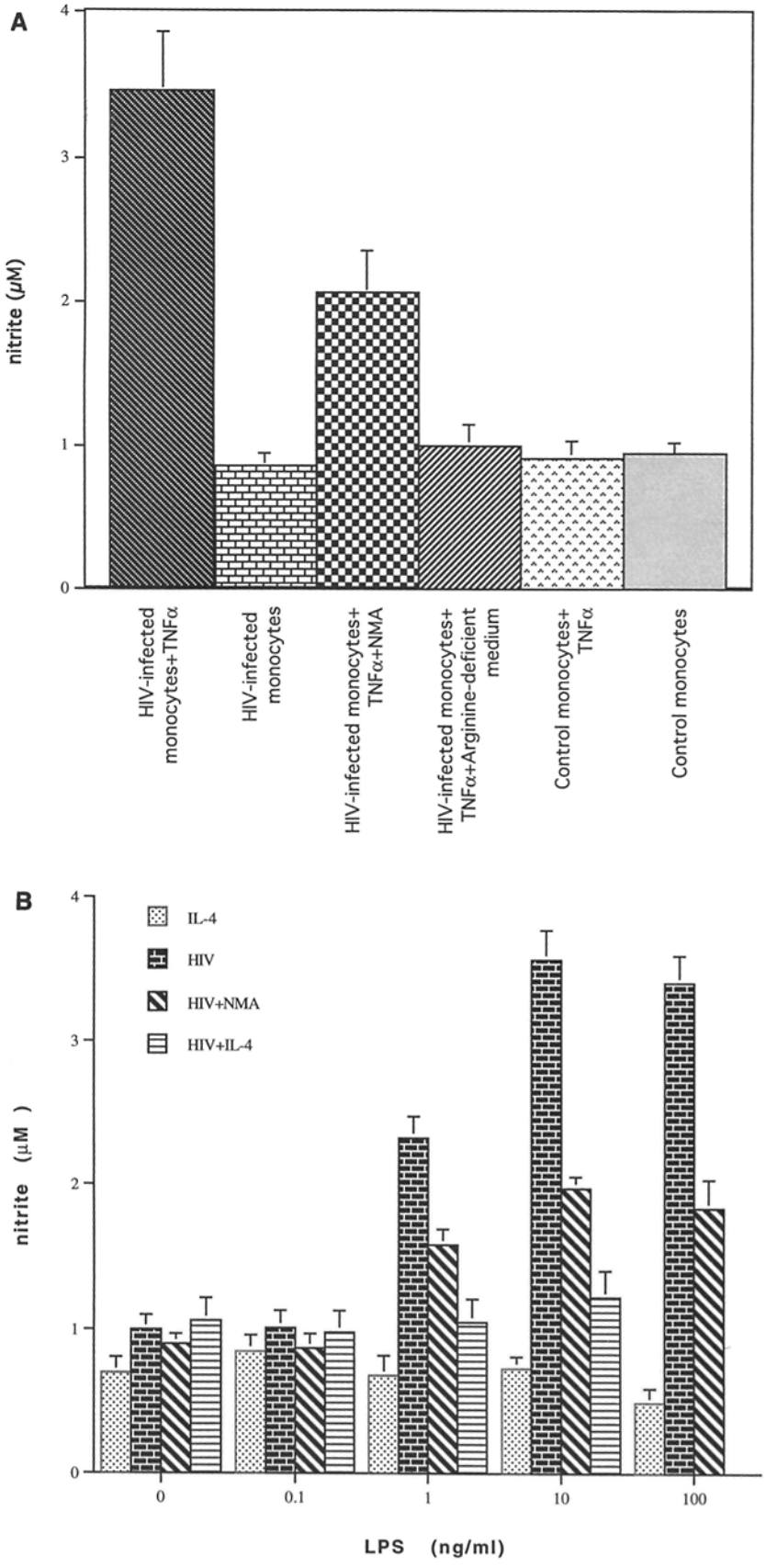

Figure 3. Positive and negative regulation of nitrite production by cultured monocytes. (*A*) 4 d after infection with HIV-1_{ADA} the medium was changed and incubation was continued for an additional 24 h in the presence of TNF-α (1,000 U/ml) with or without 2 mM NMA. Uninfected monocytes were treated in a similar way, except that the initial 4-d incubation was done without HIV. Nitrite concentration was then determined in the culture medium by the Griess reaction. (*B*) 4 d after infection with HIV-1, the medium was changed and incubation was continued for an additional 24 h in the presence of various concentrations of LPS and IL-4 (5 ng/ml) or NMA (2 mM). Uninfected monocytes were incubated with IL-4 in a similar way. For each experimental point, three independent wells with cells from the same donor were prepared. Results are presented as mean ± SD.

Table 2. *Effect of LPS Stimulation on the Nitrite Production by HIV-infected Monocytes*

Days after infection	LPS	RT (cpm/ml × 10^6)	Nitrite (μM)
5	+	0.51 ± 0.07	7.04 ± 0.02
	−	0.45 ± 0.07	4.41 ± 0.84
6	+	1.32 ± 0.04	2.37 ± 0.25
	−	1.43 ± 0.05	2.74 ± 0.24
7	+	4.42 ± 0.21	2.79 ± 0.39
	−	4.55 ± 0.16	2.40 ± 0.09

Bulk cultures of monocytes (2 × 10^6 cells/ml) in Teflon flasks were either stimulated with 10 ng/ml LPS at the indicated intervals after infection or left untreated, and after 24 h, RT activity and nitrite concentration in the culture medium were assayed. Uninfected monocytes (both LPS stimulated and unstimulated) did not produce any detectable amount of nitrites in this experiment. For each time point, three samples were withdrawn, and the results are presented as mean ± SD.

~2 mM. This value is considerably higher than the IC_{50} of 20 μM reported for mouse macrophages (33, 40, 44). In this regard it should be noted that our experiments were performed in the standard DMEM medium containing 1 mM arginine, whereas results with murine cells were obtained with 100-μM concentrations of arginine. When we lowered arginine concentration to physiologically relevant 100 μM, IC_{50} for NMA was ~100 μM (data not shown). This value is still five times higher than the IC_{50} for murine cells under similar conditions. Since NMA competes with L-arginine for both the transporter and for the NOS enzyme, the observed variation between the murine and human cells could be attributed to the differences in both these processes.

The NO· levels produced by human HIV-infected monocytes are significantly lower than in LPS-stimulated murine macrophages (19). Possibly, this species difference may be attributable to the lack of an appropriate priming and/or activating stimulus for human monocytes (26), which infection with HIV-1 provides in part. In this regard, it is notable that recent work demonstrated that HIV primes monocytes for brisk immunologic responses upon subsequent activation (Nottet, H. S. L. M., and H. E. Gendelman, unpublished results). LPS-activated HIV-1–infected promonocytic (45) and phorbol ester–activated myelomonoblastic cells (46) produce significantly more TNF-α than similarly activated uninfected control cells. Similar results are in alveolar macrophages from HIV-infected patients, implying a relationship between viral infection and the overexpression of cytokines (47). The mechanisms whereby HIV-1 primes monocytes for subsequent activation and iNOS induction are not known. gp120–CD4 interaction may play some role in priming, but HIV infection is a much more effective stimulus than gp120 treatment.

Although a recent report described high levels of NO· production by normal human monocytes after stimulation

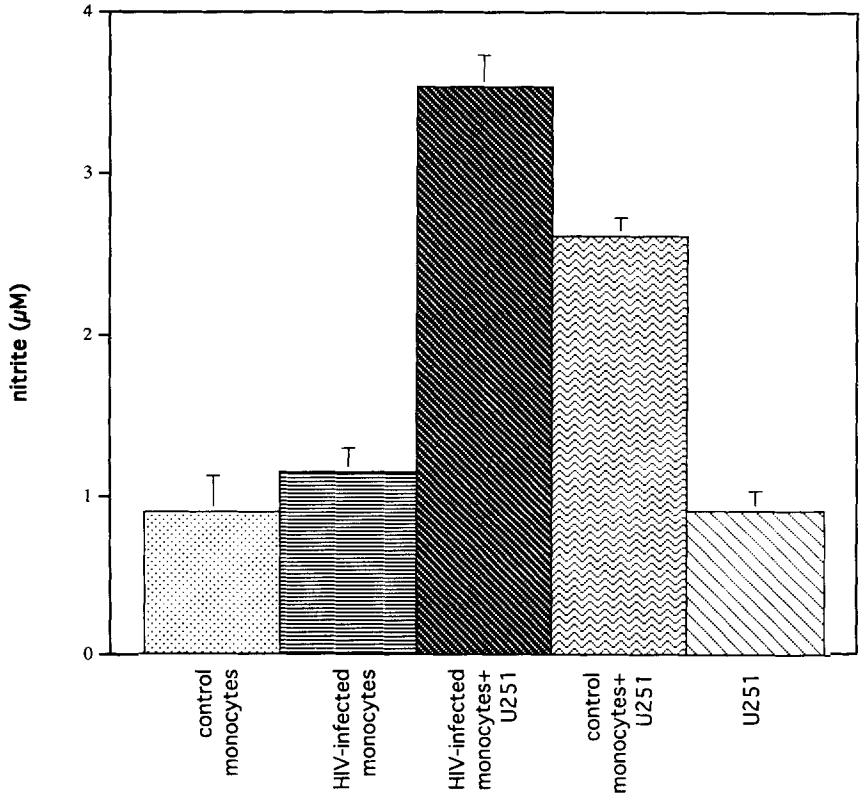

Figure 4. Nitrite production in monocyte-astroglia co-culture. 4 d after infection with HIV-1_{ADA}, U251 astroglial cells were added at a 1:1 monocyte/astroglia ratio. 48 h after initiation of co-culture, nitrite concentration was determined in culture media by the Griess reaction. Uninfected (*control*) monocytes were cultured for 4 d without HIV-1 before initiation of coculture. Experiments were done with the cells from four different donors, and results of a representative experiment are shown. For each experimental point three independent wells with cells from the same donor were prepared and assayed, and results are presented as mean ± SD.

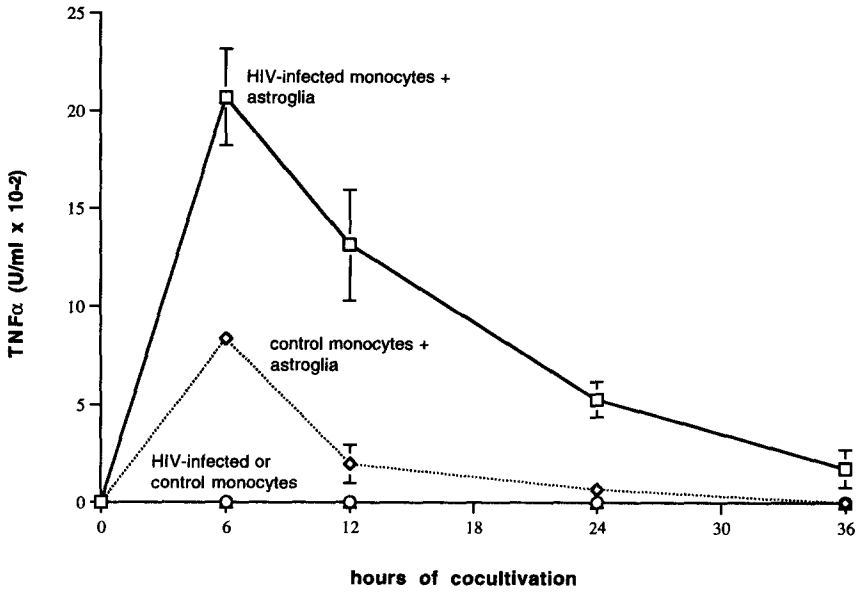

Figure 5. Induction of TNF-α in monocyte-astroglia co-cultures. Human monocyte cultures, either uninfected or infected with HIV-1_{ADA} for 4 d, were co-cultivated with U251 astroglial cells. At various times after initiation of co-culture, TNF-α was measured in the culture medium by a bioassay based on the lysis of actinomycin D–treated L929 cells (6). Each sample was measured in triplicate, and the results are presented as mean ± SD.

with LPS (29), we and other groups (24, 26, 27) did not observe these effects using cell culture conditions described. In this regard, features of monocyte cultures highly depend on the methods of cell isolation and propagation, and also demonstrate considerable donor variability. In our study, cells were cultured in the presence of M-CSF for at least 7 d before infection or stimulation, therefore were highly differentiated cells (30). We found those culture conditions optimal for accomplishing maximal levels of viral infection. We observed correlation between the $NO^{\cdot}$ production by monocytes and the level of virus replication. Usually, levels of nitrites above the background (~0.9 μM) were detected after RT activity reached 10^6 cpm/ml. However, even with high-level virus replication, there was a variability among donors. In two of six experiments where RT levels exceeded 10^6 cpm/ml, $NO^{\cdot}$ was not detected. Given the sensitivity of the Griess assay (~1 μM) and low levels of nitrite production by human monocytes (~5 μM), we cannot distinguish between low- or nonresponding cells for $NO^{\cdot}$.

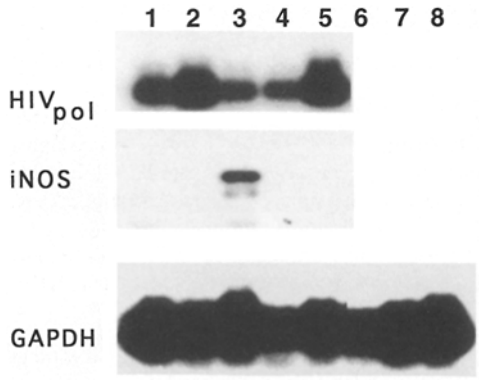

Figure 6. RT-PCR analysis of NOS RNA in brain samples. RNA extracted from brain samples (57) was reverse transcribed and analyzed by PCR with primers specific for HIV polymerase (*HIV_{pol}*), iNOS, and GAPDH sequences. Case numbers are shown above the lanes. Lanes *1–5* were from HIV-infected brains with various degrees of encephalitis, and lanes *6–8* were from uninfected brains.

When HIV-infected monocytes receive a triggering signal (which may be delivered by astroglia, TNF-α, or LPS) early in infection, iNOS activity is induced through transcription of the iNOS gene. A similar pattern was described for murine macrophages, where two signals also are required for induction of iNOS, but the first (priming) signal is provided by IFN-γ in the mouse system (44). In human monocytes, IFN-γ does not prime for iNOS transcriptional activation (data not shown). The reason for this difference between murine and human macrophages is likely due to different signal transduction pathways leading to the activation of the iNOS gene.

Given that relatively few productively infected brain macrophages are required to elicit progressive clinical deficits, HIV-induced neurotoxicity may reflect amplification of neurotoxic activities by cell activation and/or interactions between infected and uninfected cells in the brain. Recent evidence has identified not only HIV-1 gene products, but also several host proinflammatory cytokines (TNF-α, IL-1β), PAF and eicosanoids as potential neurotoxins produced by HIV-infected macrophages (5, 6). We now demonstrate nitric oxide ($NO^{\cdot}$) as an additional candidate virus-regulated neurotoxin. AIDS is associated with activation of the immune system with coincident elevated levels of TNF (8, 48, 49), a condition that may promote induction of iNOS in the infected macrophages of various tissues. Taking into consideration previously demonstrated $NO^{\cdot}$-mediated neurotoxicity associated with viral infection of the brain (21, 22, 50), it is possible that similar mechanisms operate in HIV encephalitis. The modest elevations of $NO^{\cdot}$ from HIV-infected monocytes may contribute to HIV-related neuronal injury similar to several other neurological diseases (21–23). $NO^{\cdot}$ may either directly (in conjunction with superoxide anion) damage neurons (17, 51, 52), or amplify production of other neurotoxins in a manner similar to its amplification of calcium-induced gene transcription (53).

By this mechanism, the effects of low level infection of brain macrophages could be amplified. Expression of iNOS is also a subject for negative regulation by the immune system. Our results demonstrate that IL-4 inhibits induction of NOS in LPS-stimulated HIV-infected monocytes. IL-4 was shown previously to downregulate the priming effect of IFN-γ on peripheral blood monocytes (54, 55). In addition, a neuroprotective role of IL-4 against activated microglia was recently described (56). Taken together, these data suggest a protective role for IL-4 in HIV-induced brain disease.

We were able to detect expression of iNOS in the brain tissue of a pediatric patient with severe, late-stage HIV encephalitis, indicating that iNOS can be induced during HIV-induced brain disease. The events that lead to the ADC are not yet fully understood, but probably reflect the interplay between the neurotoxic (e.g., PAF, arachidonic acid metabolites, $NO^{\cdot}$) and neuroprotective (e.g., IL-4, TGF-β) factors produced in the brain by macrophages and astroglial cells. Failure of the neuroprotective mechanisms is, most likely, the cause of uncontrolled activation associated with the development of cognitive and motor dysfunctions. The role of $NO^{\cdot}$ in these events is still unclear, since we did not observe a strong correlation between iNOS expression in the brain and AIDS dementia. It is likely that $NO^{\cdot}$ is only one of many factors leading to disease progression. Elucidation of the pathogenic role of $NO^{\cdot}$ in ADC, for instance, through experiments exploiting animal models of AIDS, may suggest new avenues for therapeutic intervention against the neurological complications of HIV infection.

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