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The regulation of quinolinic acid in human immunodeficiency virus-infected monocytes

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Quinolinic acid (Quin) is thought to underlie cognitive and motor dysfunctions for a variety of neurological disorders. Specifically, in human immunodeficiency virus (HIV)-associated dementia, Quin levels correlate with the degree of neurological dysfunction observed in affected individuals. Since recent data from our laboratories suggest that both HIV-1 infection and activation of brain macrophages are required for the development of neurotoxicity we examined Quin production during virus infection and immune activation. HIV-1 infection of monocytes induced low levels of Quin while lipopolysaccharide (LPS) or interferon-gamma (IFN-γ) activation of the virus-infected cells elicited 10-fold higher levels. The combined effects of LPS and IFN-γ for Quin production in HIV-infected monocytes was identical to each factor added alone. Little or no Quin was detected in unstimulated uninfected monocytes. LPS or IFN-γ activation of uninfected monocytes produced substantially higher levels of Quin than found in similarly stimulated HIV-1-infected monocytes. These results were at variance to the production of tumor necrosis factor-alpha (TNF-α). Here, a 2- to 5-fold increase in TNF-α levels were observed in culture fluids of LPS-activated HIV-infected cells when compared to similarly stimulated uninfected monocytes. The effect of LPS-induced Quin production by HIV-infected monocytes was not altered by primary human astrocytes. These data suggest that Quin levels seen in HIV dementia are a reflection of macrophage/microglial activation seen during advanced clinical disease. These findings could help explain, in part, why few HIV-1-infected brain macrophages can give rise to significant neurological impairments.

Keywords: AIDS dementia complex; macrophage; astrocyte; neurotoxins; cytokines

Introduction

Quinolinic acid (Quin), kynurenic acid, and L-kynurenine are neuroactive L-tryptophan metabolites that are synthesized via the kynurenine pathway. Increased concentrations of Quin are detected in serum and cerebrospinal fluid of a variety of neuropathological conditions, including the acquired immunodeficiency syndrome (AIDS) dementia complex (Heyes *et al*, 1991). As a weak agonist of N-methyl-D-aspartate (NMDA) excitatory amino acid receptors, Quin, in large concentrations, is a neurotoxin (Schwarcz *et al*, 1983; Kim and Choi, 1987). Conversely, kynumeric acid is an antagonist of excitatory amino acid receptors, including NMDA receptors. It not only protects against neurotoxic effects of Quin, but can also disrupt functions mediated through these receptors to produce neurological deficits (Foster *et al*, 1984). Macrophages and microglia, the major cellular reservoir for the human immunodeficiency virus (HIV) in brain (Koenig *et al*, 1986), are major sources for Quin production (Heyes *et al*, 1992). Although the exact mechanisms of HIV-1-induced neurological disease (Tardieu *et al*, 1992; Genis *et al*, 1992; Nottet *et al*, 1995) remains uncertain, both virus infection and immune activation of brain macrophages likely underlie progressive clinical disease (Nottet and Gendelman, 1995). Quin production by immune activated HIV-infected

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brain macrophages may, therefore, in part underlie the neurotoxic activities found in virus-infected human brain tissue (Lipton and Gendelman, 1995; Nottet and Gendelman, 1995). Astrocytes attenuate neurotoxin production by HIV-infected monocytes, but their role in HIV-1-induced brain disease is complex since depending on the level of macrophage activation primary astrocytes may reduce neurotoxin production (Nottet *et al*, 1995) or stimulate the production of neurotoxins from HIV-infected monocytes (Genis *et al*, 1992). In this context, we studied the production of Quin during HIV-1 infection and activation of human monocytes in the presence of primary human astrocytes. Astrocytes express 3-hydroxyanthranilate 3,4-dioxygenase (HAD), a Quin biosynthetic enzyme (Okuno *et al*, 1987) and contain kynurenine aminotransferase (Okuno *et al*, 1991) and can, therefore, convert the Quin metabolite kynurenine to kynurenine acid, a broad spectrum antagonist of excitatory amino acid receptors (Foster *et al*, 1984), thereby interfering with macrophage neurotoxic activities. Thus, the investigation of Quin synthesis during immune activation of monocytes following HIV-1 replication and astrocyte cell-to-cell interactions could provide an insight into its regulation within the brains of virus-infected subjects.

Results

A laboratory model system to study Quin production in monocytes was utilized to reflect what may occur in virus-infected brain macrophages *in vivo*. Four days after HIV-1_{ADA} exposure levels of RT activity and Quin were measured. As shown in Figure 1, levels of RT activity paralleled low levels of Quin in the HIV-1 infected cultures. HIV-1 infection was thus a poor inducer of Quin production in monocytes. The increases in [^{12}C]-Quin were identical to the increase in [^{2}H]-Quin when the media contained [^{2}H]-tryptophan (data not shown), suggesting that the HIV-1-induced Quin production in monocytes was due to increased *de novo* production from tryptophan. Replicate cells inoculated with heat-inactivated HIV-1, produced no Quin indicating that Quin induction depended on productive viral replication. HIV-1 infection of monocytes (in five replicate experiments), however, did not result in TNF-α production. These latter results are in agreement with our previously published reports (Gendelman *et al*, 1990, Nottet *et al*, 1995).

Since both viral infection and immune activation of brain macrophages likely play a role in HIV neuropathogenesis (Nottet and Gendelman, 1995), the effect of HIV-1 infection and immune stimulation of monocytes on the production of Quin by monocytes was determined. Monocytes were inoculated with HIV-1 then stimulated 1, 3 and 4 days after viral infection with LPS (Figure 2). Monocytes infected for 1 day produced equal amounts of Quin after LPS stimulation than replicate uninfected cells (Figure 2A). These levels were up to 10-fold higher than observed in unstimulated HIV-1-infected cells. Interestingly, 3 and 4 days after viral infection, the HIV-1-infected cells produced lower levels of Quin than uninfected cells following LPS stimulation (Figure 2B and C). In contrast, the amount of TNF-α produced by LPS-stimulation HIV-1-infected monocytes was 2-5 fold higher than stimulated uninfected monocytes (Figure 2E and F). The values shown are representative of four separate experiments. Each data point represented an average of quadruplicate determinations with s.d. shown by error bars.

To further investigate the effects of cellular activation on Quin production monocytes, cells were infected with HIV-1_{ADA} for 4 days and then stimulated with 5 ng/ml LPS, 100 U/ml IFN-γ or both used in combination. IFN-γ stimulation of uninfected monocytes resulted in higher levels of Quin production than in IFN-γ stimulation of HIV-1-infected monocytes (Figure 3a). Importantly, stimulation of uninfected or HIV-infected monocytes with both IFN-γ and LPS did not result in increased Quin production over what was found in IFN-γ-stimulated cells alone (Figure 3b). This suggested that the LPS and IFN-γ stimulate Quin production in monocytes by a common intracellular mechanism. Interestingly, IFN-γ stimulated HIV-1-infected monocytes did not secrete TNF-α while the combined treatment of IFN-γ and LPS had a greater effect on TNF-α production by uninfected monocytes than on HIV-infected monocytes (data not shown).

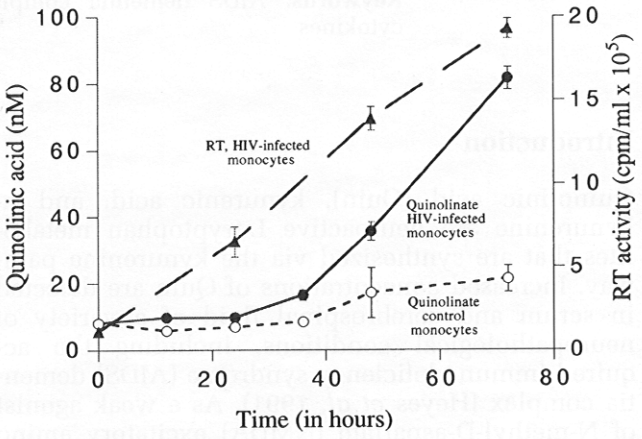

Figure 1 Quin levels and RT activity levels in culture fluids of HIV-1_{ADA} and uninfected monocytes. PBMCs from HIV and hepatitis B seronegative donors were purified by leukopheresis and countercurrent centrifugal elutriation to >98% monocytes. The monocytes were cultured 7 days as adherent monolayers and then exposed to HIV-1_{ADA} at an MOI of 0.1. At specified hours after infection media was replaced with fresh media and aliquots were tested for RT activity and Quin.

Since astrocytes have been demonstrated to affect neurotoxin production by HIV-infected monocytes (Nottet *et al*, 1995), we added primary human astrocytes at a 1:1 ratio with replicate monocytes 4 days after HIV-1 monocyte infection. Basal and LPS-induced release of Quin was measured at increasing times thereafter. Astrocytes did not alter Quin production by either HIV-infected (Figure 4) or uninfected monocytes. In addition, the amount of Quin released by HIV-infected or uninfected LPS-stimulated monocyte-astrocyte cocultures was similar to that of the replicate stimulated HIV-infected (Figure 4) or uninfected monocytes. In contrast, our previous works demonstrated that astrocytes downregulated the LPS-induced TNF-α overexpression by HIV-infected monocytes (Nottet *et al*, 1995). These data, together, suggest that the regulatory pathways for Quin and TNF-α in virus-infected and control uninfected monocytes are distinct. Most importantly the data demonstrate that cellular activation rather than HIV-1 infection is a better stimulus for Quin production.

Discussion

The present data indicate that activation of brain macrophages could account for increases in Quin seen in HIV-associated dementia. Addition of astrocytes to monocyte cultures did not alter monocyte secretion of Quin. These results are consistent with a previous study reporting that human fetal brain tissue readily produce L-kynurenine but no Quin (Heyes *et al*, 1993). Furthermore, the findings reported by Heyes *et al* (1993) suggested that macrophage infiltration in the brain parenchyma and subsequent cellular activation resulted in elevated Quin levels within the CNS, a conclusion that is supported by this study.

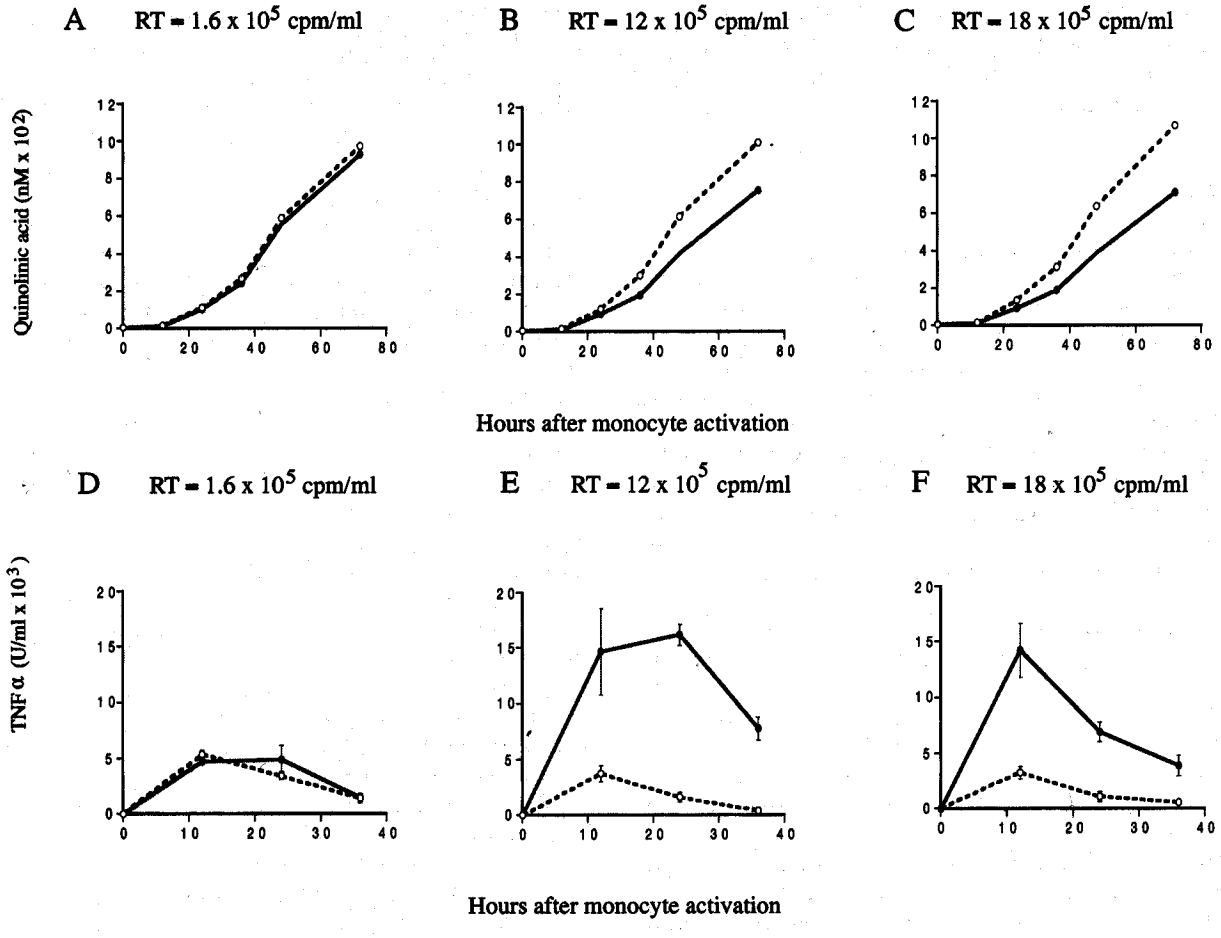

Figure 2 Quin and TNF-α bioactivity levels in culture fluids of LPS-stimulated HIV-1_{ADA}-infected and uninfected control monocytes. PBMCs from HIV-seronegative donors were purified to >98% monocytes, cultured 7 days as adherent monolayers and then exposed to HIV-1_{ADA} at an MOI of 0.1. At 1 (**A, D**), 3 (**B, E**) and 4 (**C, F**) days after infection, HIV-1-infected monocytes (solid lines and circles) and control uninfected monocytes (open circles, dotted lines) were stimulated with 5 ng/ml LPS. Culture fluids were removed and levels of Quin (**A, B, C**) and TNF-α activity (**D, E, F**) were assayed. Results are expressed as mean ± s.d. of quadruplicate determinations.

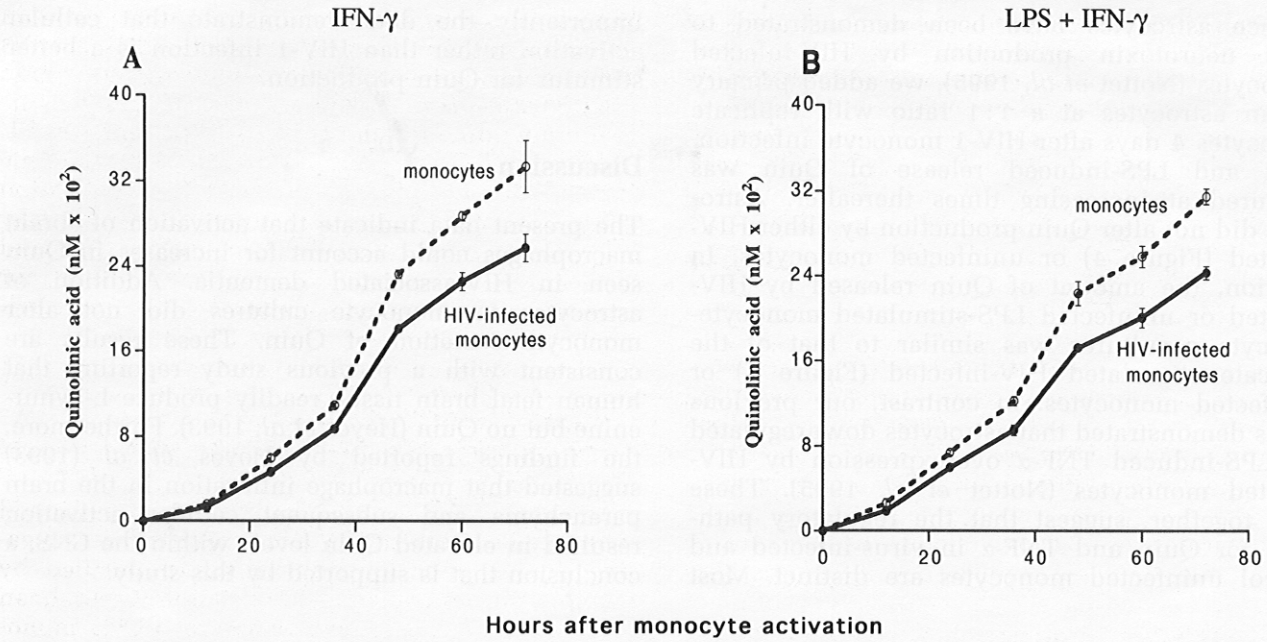

Figure 3 Quin levels in culture fluids of activated HIV-1_{ADA}-infected and uninfected control monocytes. PBMCs from HIV-seronegative donors were purified to >98% monocytes, cultured 7 days as adherent monolayers and then exposed to HIV-1_{ADA} at an MOI at 0.1. Four days after infection, media was replaced with fresh media and HIV-1-infected monocytes (solid lines and circles) and control uninfected monocytes (open circles, dotted lines) were stimulated with 100 U/ml IFN-γ alone (**A**) and with 5 ng/ml LPS and 100 U/ml IFN-γ (**B**). Culture fluids were removed and levels of Quin were assayed. Results are expressed as mean $\pm$ s.d. of quadruplicate determinations.

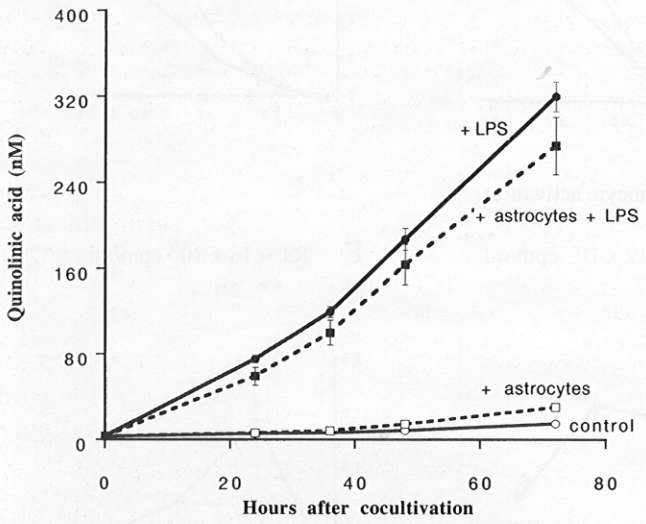

Figure 4 Quin levels in culture fluids of activated HIV-1_{ADA}-infected monocytes cocultured with primary human astrocytes. PBMCs from HIV-seronegative donors were purified to >98% monocytes, cultured 7 days as adherent monolayers and then exposed to HIV-1_{ADA} at an MOI of 0.1. Four days after infection, cells were unstimulated (open symbols) or stimulated with 5 ng/ml LPS (closed symbols) and astrocytes were added at a 1:1 monocyte-astrocyte ratio to replicate cultures (dotted lines). Culture fluids were removed at various intervals and levels of Quin were assayed. Results are expressed as mean $\pm$ s.d. of quadruplicate determinations and is representative of two independent experiments with different monocyte donors.

In this report, we demonstrate that Quin is produced at low levels following HIV-1 infection of monocytes. Subsequent stimulation of these cells with either LPS or IFN-γ results in lower Quin levels than similarly treated replicate uninfected cells. In contrast, LPS-activated HIV-infected monocytes produced higher levels of TNF-α than did uninfected cells. These findings demonstrate that the regulation of Quin and TNF-α is markedly different in HIV-infected monocytes. The role of viral infection in Quin production by monocytes is seemingly less important than cellular activation.

Our initial works demonstrated that one means for macrophage activation is through cell to cell interactions with astroglial cells. We found that these cells can cooperate in the production of a number of putative neurotoxins which include eicosanoids, platelet activating factor (PAF), nitric oxide (NO) and proinflammatory cytokines (IL-1β, TNFα, etc. (Genis *et al*, 1992; Gelbard *et al*, 1994; Bukrinsky *et al*, 1995; Nottet *et al*, 1995, 1996). An important observation centered around the notion that once the macrophage becomes HIV-infected, its response to activation becomes altered so that immune stimulation results in several times greater levels of 'putative neurotoxins' than found in replicate uninfected cells similarly affected. In this way, HIV likely primes the macrophage for subsequent immune activation. Other activating stimuli

were soon sought and included LPS and opsonized zymosin (Opz) (Nottet *et al*, 1995 and Flanagan *et al*, unpublished observations). In some ways this finding mirrored those exerted by IFN-γ. Primed macrophages produce high levels of many effector molecules; significantly higher than what is seen without such immunologic priming. Therefore, the use of LPS in this report was a tool to produce immunologic competence in macrophages. Like many *in vitro* experimental models proposed for studies of HIV-1 neuropathogenesis (Pulliam *et al*, 1991, 1993; Giulian *et al*, 1991; Brenneman *et al*, 1988; Dreyer *et al*, 1990; Moses *et al*, 1993) this system has its own set of limitations in reflecting the exact *in vivo* condition. First, LPS is not the immune trigger seen by the brain macrophage/microglia during induction of disease. Second, LPS may give rise to a set of immunological responses distinct from those demonstrated in the brain during HIV-1-infection. Third, single cell infections with monocytes cannot mimic the complexities of brain macrophage/microglial-neuronal cell-cell interactions, neuroimmune regulation and the plethora of co-infections seen in the CNS during advancing virus-induced neurological impairments. Fourth, studies performed with peripheral blood monocytes may not always be directly relevant to CNS macrophages/microglia. Nevertheless, and on balance, the data shown does provide several important insights into the abilities of the monocyte-derived macrophage (critical target cells for HIV-1 in brain) to regulate QUIN. The low levels of Quin produced by HIV-1-infection alone and its significant augmentation after immune activation suggest that both viral infection and immune activation is required for brain disease (Glass *et al*, 1995). This idea supports recent observations regarding the levels of QUIN seen in the CNS during advancing neurological impairment of affected individuals (Sei *et al*, 1995).

What role then does Quin play in the pathogenesis of HIV-associated cognitive/motor complex? In our previously published *in vitro* models for HIV-1 encephalitis, we demonstrated that both HIV-1 infection and macrophage stimulation were required for neurotoxin production (Nottet *et al*, 1995; Nottet and Gendelman, 1995). Indeed, there is ample evidence for diffuse CNS activation in brain tissue of patients with HIV-1-associated neurological impairments (Wesselingh *et al*, 1993; Nottet *et al*, 1996). The finding that high level viral gene expression in brain tissue does not always correlate with clinical manifestations of neurological impairment (Kure *et al*, 1990; Wiley and Achim, 1994), emphasizes the importance of macrophage activation in the pathogenesis of HIV-associated cognitive/motor complex. Our findings suggest that brain and CSF Quin levels observed in HIV-infected individuals (Heyes *et al*, 1991; Sei *et al*, 1995) might be a reflection of immune activation of brain tissue rather than a consequence of direct viral replication. These data suggest that the increased Quin levels observed in CSF of demented AIDS patients are a reflection of uninfected macrophage activation seen during advanced clinical disease. The finding that zidovudine treatment of AIDS patients results in lower CSF Quin levels (Heyes *et al*, 1991) and the findings in this report suggest that HIV-1 replication may affect macrophage activation. This study may help explain how few HIV-1 infected cells could give rise to the devastating cognitive and motor impairments seen in neurologically affected individuals with AIDS.

Materials and methods

Isolation and culture of primary human monocytes and astrocytes

Monocytes were recovered from peripheral blood mononuclear cells of HIV and hepatitis B seronegative donors after leukopheresis and purified by counter current centrifugal elutriation (Gendelman *et al*, 1988). 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 48 mm plastic culture wells) in DMEM (Sigma Chemical Co., St. Louis, MO) with 10% heat-inactivated pooled human serum, 50 μg/ml gentamicin (Sigma), 10 μg/ml ciprofloxacin (Sigma) and 1000 U/ml highly purified recombinant human MCSF (a generous gift from Genetics Institute, Inc., Cambridge, MA). All tissue culture reagents were screened before use and found negative for endotoxin (<10 pg/ml; Associates of Cape Cod, Inc., Woods Hole, MA) and mycoplasma (Gen-probe II; Gen-probe Inc., San Diego, CA).

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 Nebraska Medical Center guidelines) as described previously (Nottet *et al*, 1995). Briefly, brain tissue composed of telencephalon with both cortical and ventricle surfaces was dissected in cold HBSS with 25 mM HEPES and 50 μg/ml gentamicin, then transferred to 20 ml ice-cold DMEM/F12 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated FCS. The tissue was mechanically dissociated by teasing through a Nitex bag and by filtration through a 230 and 140 μm sieve. The cell suspension was centrifuged, washed twice in media, then plated in DMEM/F12 containing 10% heat-inactivated FCS, 50 μg/ml penicillin and steptomycin, 100 μg/ml neomycin and 2.5 μg/ml fungizone (Gibco) into 150 cm^2 tissue flasks (Corning, Corning, NY) at a cell density of 10^6 cells/ml. Nonadherent microglia and oligodendrocytes were removed by circular shaking of cultured cell preparations 10 days after plating. Cells were then

cultured in DMEM/F12 with 10% heat-inactivated FSC, 20 μg/ml gentamicin and 10 μg/ml ciprofloxacin. The purity of the cell preparations was shown to be >99% astrocytes (Nottet *et al*, 1995).

HIV-1 infection of macrophages

Monocytes cultured for 7 days were exposed to HIV-1_{ADA} (Gendelman *et al*, 1988) at a multiplicity of infection (MOI) of 0.1 infectious virus/target cell. All viral stocks were found to be free of mycoplasma (Gen-probe II; Gen-probe Inc., San Diego, CA) and endotoxin (<10 pg/ml; Associates of Cape Cod). Culture medium was half-exchanged every 3 days. Reverse transcriptase (RT) activity was determined in triplicate samples of culture fluids that were 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 $MgCl_2$ and ^{3}H-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 95% ethanol in an automatic cell harvester (Skatron, Inc., Sterling, VA) on paper filters. Radioactivity was estimated by liquid scintillation spectroscopy (Gendelman *et al*, 1990).

Chemical reagents and cytokines

LPS, obtained from *Pseudomonas aeruginosa* serotype 10, was purchased from Sigma and used at a concentration of 5 ng/ml. Recombinant human interferon-γ (IFN-γ) was purchased from Amgen (Thousand Oaks, CA) and used at a concentration of 100 U/ml.

Quantitation of cytokine activity

TNF bioactivity was performed according to standard procedures (Nottet *et al*, 1995). Briefly, the murine L929 cell line was propagated in DMEM (Sigma), 5% FCS, 2 mM glutamine, and 20 μg/mL gentamicin. Cells were retrieved in log phase and placed (0.7×10^5 well) in 96-well plates (Costar) with actinomycin D (Sigma). Culture fluids were inoculated into cell monolayers, and degree of cell lysis was determined by crystal violet staining after a 24 h incubation. To substantiate that the bioactivity measured was TNF-α a neutralizing TNFα antibody kindly provided by Dr J Verhoef, University of Utrecht (The Netherlands) was employed in assay mixtures of select experiments. The addition of this antibody abrogated TNF activity in the bioassay.

Quantification of Quin

Quin was measured according to the methods of Heyes and Markey (1988) with modifications as described below. Aliquots of tissue culture supernatant or cerebrospinal fluids (100 μl) were added to 1 ml of 50 mM of Tris-HCl (pH 7.5) containing 62.5 pmol [$^{13}C_7$]-Quin (>99.99% ^{13}C, Cambridge Isotope Laboratories, Woburn, MA) as internal standard. Samples were heat-inactivated at 95°C for 10 min and centrifuged for 5 min at 14 000 ×g to precipitate proteins. Aliquots (800 μl) of the supernatant were extracted with an equal volume of chloroform and then applied to weak anion exchange resins, washed with 50 mM Tris buffer and methanol and then eluted with 2 M formic acid. The eluates were dried in a vacuum centrifuge and derivitized with hexafluro-2-propanol using trifluoroactylimidazole as a catalyst for 60 min at 95°C. Samples were extracted with 100 μl heptane and 350 μl water. These extracts were then separated on a DB-1 column (0.32 ID, 30 m length with a 0.25 μm coating thickness; J&W Scientific, Folsom, CA) in a gas chromatograph and quantified using a mass spectrometer (TRIO-1; Fisons, Dearbon, MA). The mass spectrometer was operated in the negative chemical ionization mode using methane. Endogenous and [^{13}C]-Quin (internal standard) were monitored in the single ion mode as ions with m/z of 467 and 474, respectively. In some experiments, [^{2}H]-tryptophan was added to the medium and the corresponding isotopomer of Quin was measured at a m/z of 470.

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