



Quinolinic acid production is related to macrophage tropic isolates of HIV-1

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We sought to determine whether the neurotoxin quinolinic acid (QUIN) was produced by macrophages or lymphocytes infected with isolates of HIV-1 with varying degrees of macrophage tropism derived from patients with varying stages of AIDS dementia complex (ADC). Highly macrophage tropic isolates and minimally macrophage tropic isolates were used to inoculate macrophages and QUIN production was measured. Similarly, QUIN production from macrophages was monitored using a purified cell free highly macrophage tropic isolate and laboratory isolates SF₃₃ and SF₂. Each of these experiments was also performed with lymphocytes. We found that macrophages infected with macrophage tropic isolates of HIV-1 led to QUIN production while lymphocytes did not produce QUIN. The ability of the HIV-1 infected macrophages to produce QUIN was related to the viral inoculum and the degree of macrophage tropism of the isolate. The severity of ADC in the patient from whom a particular isolate was derived was not *per se* a determining factor for QUIN production. Purified cell free ADC isolates also led to QUIN production by macrophages thereby suggesting that HIV-1 infection alone is capable of inducing QUIN production.

Keywords: quinolinic acid; excitotoxin; macrophages; AIDS dementia; HIV

Introduction

QUIN is a neurotoxin, acting through the N-methyl D-aspartate (NMDA) receptor on neurons (Schwarcz *et al*, 1983; Kim and Choi, 1987; Whetsell and Schwarcz, 1989), that is potentially important in the pathogenesis of ADC. QUIN is present in raised concentrations in the cerebrospinal fluid (CSF) of patients with ADC (Heyes *et al*, 1991). Moreover, several investigators have observed neuronal loss in patients with ADC (Ketzler *et al*, 1990; Wiley *et al*, 1991), a finding which would be consistent with the presence of a neurotoxin. However, the identity of the cells that are producing QUIN is unknown. Because HIV-1 can productively infect macrophages and lymphocytes and γ interferon stimulated macrophages can produce QUIN (Heyes *et al*, 1992), we hypothesized that HIV-1 infected macrophages, lymphocytes or both would lead to QUIN production and that there would be greater QUIN produc-

tion by cells infected with isolates of HIV-1 from demented as opposed to non-demented patients.

Results

From experiment #1 macrophages infected with an ADC isolate produced significantly more QUIN than macrophages infected with a non-ADC isolate at each of the time points ($P < 0.0001$) (Figure 1). *In situ* hybridization for HIV-1 mRNA at 24 h confirmed productive infection with approximately 12% of the macrophages inoculated with the 4⁺ macrophage tropic isolate being positive while 2% of the macrophages inoculated with the 1⁺ macrophage tropic isolate were positive. Experiment #2 revealed that increasing inocula of another ADC isolate led to progressively increasing amounts of QUIN (Figure 2). No QUIN was detected from macrophages infected with the same inocula of HIV-1 from a patient without ADC whose isolate was much less macrophage tropic. In experiment #3 it was then shown that infection of macrophages with the purified cell free ADC isolate led to similar

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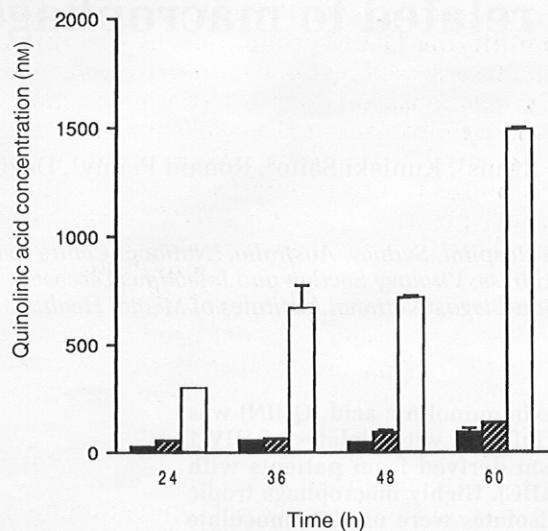


Figure 1 [$^{13}\text{C}_6$]-QUIN production by macrophages infected with isolates of HIV-1 from a patient with severe ADC (stage 3) (4 $^+$ macrophage tropic HIV-1 isolate) and no ADC (stage 0) (1 $^+$ macrophage tropic HIV-1 isolate): column 1 represents macrophages mock infected with SUPT1 supernatant, column 2 macrophages infected with a non-ADC HIV-1 isolate and column 3 macrophages infected with an ADC isolate. Results are expressed as means and standard error of the mean of triplicate experiments.

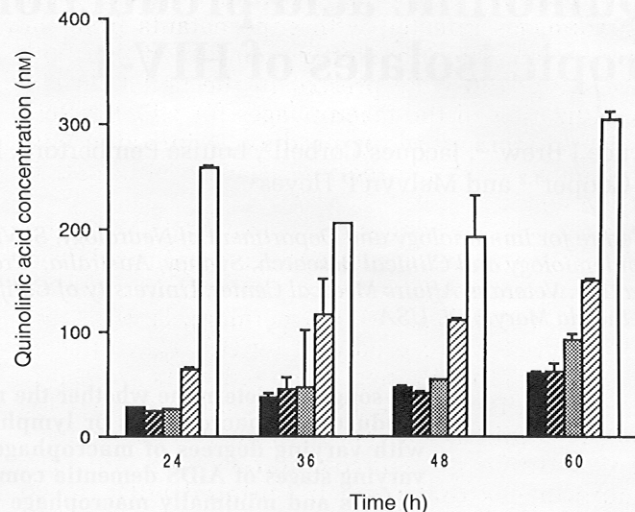


Figure 2 [$^{13}\text{C}_6$]-QUIN production by macrophages infected with increasing TCID₅₀/ml of HIV-1 from a patient with ADC whose isolate was 3 $^+$ macrophage tropic: column 1 represents macrophages mock infected with SUPT1 supernatant, column 2 macrophages infected with a macrophage tropic ADC isolate of HIV-1 at 20 TCID₅₀ ml⁻¹, column 3 at 40 TCID₅₀ ml⁻¹, column 4 at 200 TCID₅₀ ml⁻¹ and column 5 at 400 TCID₅₀ ml⁻¹. Results are expressed as means and standard error of the mean of triplicate experiments.

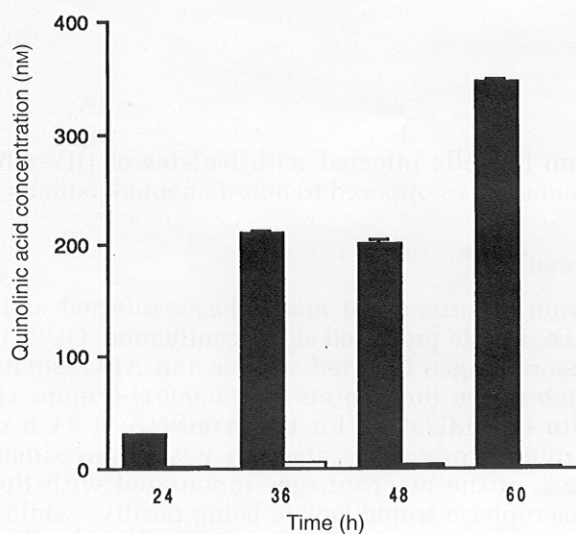


Figure 3 [$^{13}\text{C}_6$]-QUIN production by macrophages infected with a 2 $^+$ macrophage tropic isolate of HIV-1 from a patient without ADC as opposed to macrophages infected with a 1 $^+$ macrophage tropic isolate from a patient with ADC: column 1 macrophages infected with a non-ADC but macrophage tropic isolate of HIV-1 and column 2 macrophages infected with an ADC isolate with low macrophage tropism. Results are expressed as means and standard error of the mean of triplicate experiments.

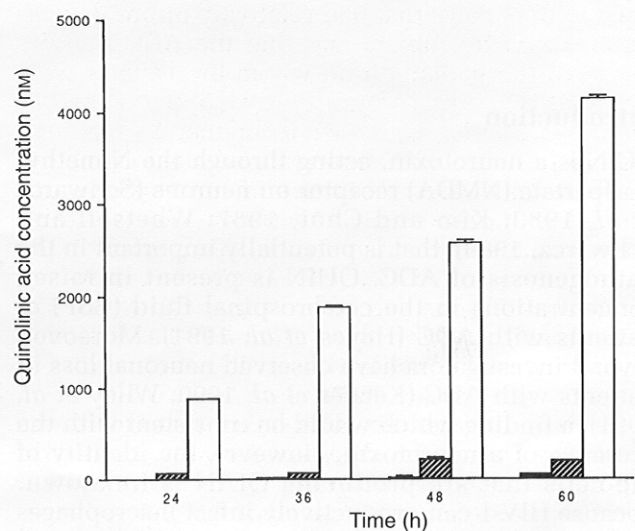


Figure 4 [$^{13}\text{C}_6$]-QUIN production from [$^{13}\text{C}_6$]-tryptophan by macrophages infected with HIV-1_{SF2} and HIV-1_{SF33} compared to mock infected (SUPT1 supernatant) macrophages: column 1 represents macrophages mock infected with SUPT1 supernatants, column 2 represents macrophages infected with HIV-1_{SF2} and column 3 macrophages infected with HIV-1_{SF33}. Results are expressed as means and standard error of the mean of triplicate experiments.

amounts of QUIN as were produced from macrophages infected with supernatants from the ADC isolate. Immunoblotting confirmed that both p24 and gp120 were present in the pellet. *In situ* hybridization of the macrophages for HIV-1 mRNA was positive confirming productive infection with the same rates of infection as were obtained in experiment #1.

In experiment #4 there was greater QUIN production from the more macrophage tropic isolate of HIV-1, even though it was isolated from a patient without ADC (Figure 3). Furthermore, large quantities of QUIN were produced by HIV-1_{SF33} infected macrophages while negligible amounts were produced by HIV-1_{SF2} (Figure 4). *In situ* hybridization for HIV-1 mRNA for the different isolates showed the following: approximately 5% of the macrophages that were inoculated with the 2⁺ macrophage tropic isolate were positive, 2% for the 1⁺ isolate, 12% for the SF₃₃ isolate and 2% for the SF₂ isolate.

Lymphocytes did not produce detectable amounts of QUIN at any of the time points when infected with any of the isolates. Similarly, lymphocytes infected with HIV-1_{SF2} did not produce detectable amounts of QUIN.

Discussion

We have shown that macrophages acutely infected with macrophage tropic HIV-1 isolates produced significantly more QUIN than macrophages inoculated with isolates that had relatively minor degrees of macrophage tropism and that the ADC severity *per se* of the patients from whom the isolates were derived did not influence QUIN production. Moreover, similar results were obtained by inoculating macrophages with laboratory adapted isolates of HIV-1 with differing degrees of macrophage tropism. Furthermore, our experiments to minimize the influence of cellular factors such as cytokines have demonstrated that HIV-1 infection *per se* of macrophages is capable of leading to QUIN production. Finally, we have shown that HIV-1 infection of lymphocytes does not lead to QUIN production.

While only seven HIV-1 isolates were used in these experiments, the observation that QUIN was produced by several different isolates, both wild type and laboratory adapted, makes it likely that other macrophage tropic isolates of HIV-1 are capable of QUIN production. That QUIN production was related to significant HIV-1 macrophage infection *per se* rather than macrophage related cytokines is supported by (1) the observation that QUIN was still produced by macrophages infected with the purified cell free HIV-1 isolate and by (2) the lack of QUIN production by HIV-1 isolates that were only 1⁺ macrophage tropic — that is isolates that were capable of producing more than 500 pg ml⁻¹ of p24 at one time point when inoculated into

macrophages. However, the importance of cytokines in amplifying QUIN production by macrophages cannot be assessed by these current experiments.

The relationship between QUIN production and macrophage tropism of HIV-1 is supported directly by the results of the last experiment where a macrophage tropic isolate from a non-demented patient produced more QUIN than a less macrophage tropic isolate from a demented patient. Moreover, indirect evidence of the relationship is implied by the lower amounts of QUIN produced in experiment #4 (Figure 3) compared to experiment #1 (Figure 1) perhaps because of the lower (2⁺ as opposed to 4⁺) degree of macrophage tropism of the isolate.

Our finding of QUIN production by macrophages is in contrast to Giulian *et al* (1990) who noted that there was no significant QUIN production from the HIV-1 infected monocytoïd cells that they studied. It should be noted, however, that Giulian *et al* studied a chronically infected cell line while this study examined the effects of acute infection in primary cells. Moreover, it is apparent from our study that macrophage QUIN production is a function of the viral inoculum and the degree of macrophage tropism of the isolate.

The association between QUIN production and HIV-1 macrophage tropism is in accord with other work on the pathogenetic importance of macrophage tropism in ADC. *In vitro* data have shown that only macrophage tropic strains of HIV-1 can infect primary brain cultures (Jordan *et al*, 1991) and *in vivo* the only productively infected neural cell in the brains of patients with ADC is the microglial cell, the ontogenetic equivalent of the macrophage (Michaels *et al*, 1988; Budka, 1990). Some investigators have demonstrated productive infection in other neural cells such as the astrocyte, oligodendrocyte, endothelial cell and even neuron; however, these data have not been consistently replicated and at present remain controversial (Price, 1995).

The lack of association between QUIN production and ADC severity of the patients from whom the HIV-1 isolates were derived does not necessarily mitigate against the potential pathogenetic importance of QUIN. Macrophage tropism is known to vary according to the donor macrophage cells that are used so that the same isolate may have differing degrees of macrophage tropism with different donor macrophage cells (Fouchier *et al*, 1994; Brew *et al*, unpublished observations). It is therefore possible that the ADC isolates that were not macrophage tropic in these experiments would be macrophage tropic if they were inoculated into other macrophages and perhaps into macrophages that had been derived from patients with ADC.

QUIN production by HIV-1 infected macrophages may partly explain the observation that there are relatively few inflammatory infiltrates with

macrophages and multinucleated giant cells and relatively little productive infection of the brain for the severity of the clinical deficit (Glass *et al*, 1993; Brew *et al*, 1995). Firstly, HIV-1 infection of macrophages in the systemic circulation will lead to the production of QUIN intermediates capable of crossing the blood brain barrier and being metabolised to QUIN by astrocytes that have been activated by cytokines produced systemically and locally in the brain (Heyes MH *et al*, 1991). Secondly, as has been demonstrated in these experiments, relatively small numbers of macrophages infected with highly macrophage tropic isolates of HIV-1 will produce large quantities of QUIN which in turn are capable of leading to considerable neuronal loss.

The concentrations of QUIN noted in these experiments are likely to be biologically relevant as they exceed the concentrations that are known to be neurotoxic in rat brain (Schwarcz *et al*, 1983). Secondly, the QUIN concentrations are of the same order as those observed by us to occur *in vivo* in the CSF of patients with ADC (Heyes *et al*, 1991). Thirdly, we have demonstrated that larger concentrations of QUIN are produced by isolates from demented as opposed to non-demented patients, provided the isolates are macrophage tropic, in keeping with our *in vivo* observations of higher CSF QUIN concentrations in demented patients.

These data demonstrate that HIV-1 infected macrophages are an important source of QUIN and that QUIN has a unique role in ADC by virtue of its production being linked to the degree of macrophage tropism of the particular isolate of HIV-1.

Materials and methods

Patients

ADC was diagnosed in those patients who had characteristic symptoms and signs and in whom CT brain scan, cerebrospinal fluid analysis, blood count, chemistry, vitamin B₁₂ and folate levels excluded other potential diagnoses. Neuropsychological assessment was performed to confirm the diagnosis. The severity of ADC was graded using the Price Brew scale (Price and Brew, 1988). There were three patients with ADC and three patients without ADC.

Preparation of HIV-1 isolates

Isolates of HIV-1 were obtained by coculture of peripheral blood mononuclear cells (PBMCs) from the demented and non-demented HIV-1 infected patients with PHA stimulated PBMCs from HIV-1 seronegative donors. Cultures were assessed for productive HIV-1 infection by monitoring for release of p24 into the culture supernatant (Genetic Systems) every 3 days for 30 days. Supernatants that were found to be positive for p24 were then used to inoculate

10 × 10⁶ PHA stimulated PBMCs for stock virus preparation. Subsequently, supernatants with > 500pg ml⁻¹ of p24 were stored at - 70°C in 4 ml aliquots. Viral titres were determined using the Karber method (Karber G, 1931).

Preparation of macrophages

Human macrophages were isolated from the peripheral blood mononuclear cells of a healthy HIV-1 seronegative donor by adherence (Cheng-Mayer *et al*, 1990; Koyanagi *et al*, 1987; Pulliam *et al*, 1991); the same donor was used for each experiment. Monoclonal antibody staining with CD68 confirmed that most (> 90%) of the cells were macrophages. The cells were then maintained in RPMI medium supplemented with 10% human serum, 10% glutamine, penicillin (100µg ml⁻¹)/streptomycin (100U ml⁻¹) and HEPES (25mM).

Determination of the degree of macrophage tropism of the HIV-1 isolates

Macrophages were inoculated with 100 TCID₅₀ of the various HIV-1 isolates from the ADC and non-ADC patients. The macrophages were incubated overnight before being washed three times and cultured in RPMI-1640 with 10% human serum. Productive infection of these macrophages was again assessed by p24 production in the supernatant every 3 days for 30 days. The isolates were then characterized according to the degree of macrophage tropism by the number of times at 3 day intervals the p24 supernatant concentration exceeded 500 pg ml⁻¹: 1⁺ = one point > 500 pg ml⁻¹, 2⁺ = two time points > 500 pg ml⁻¹, 3⁺ = three time points > 500 pg ml⁻¹ and 4⁺ = four time points > 500 pg ml⁻¹.

Preparation of final macrophage and control cultures

After having determined the macrophage tropism of the HIV-1 isolates, further macrophages were isolated as previously described and maintained in RPMI medium supplemented with 10% human serum, 10% glutamine, penicillin (100 µg ml⁻¹)/streptomycin (100U ml⁻¹) and HEPES (25mM) for 7 days. The cultures were transferred to RPMI plus the serum free medium AIMV (Gibco Chemicals, Life Technologies Inc., Grand Island, NY, USA) (1:1) for 3 days and finally placed in AIMV medium alone for an additional 3 days. [¹³C₆]-tryptophan was added to AIMV medium: if [¹³C₆]-QUIN was detected then it would prove that it had been synthesised from [¹³C₆]-tryptophan by the kynurenine pathway: 27.6mg of [¹³C₆]-tryptophan was diluted in 10 ml distilled water and 1ml was added to 50 ml AIMV medium to give a final concentration of 0.055 mg ml⁻¹ of [¹³C₆]-tryptophan in AIMV medium. SUPT1 cells, a T cell line, were used as a control cell line and grown by the same methods. All cultures were regularly screened for endotoxin (Limulus

Amebocyte Lysate Pyrotell Multitest vial, Pyrotell Associates of Cape Cod Inc., PO Box 224, Woods Hole, MA, USA) and mycoplasma (Gen-Probe Mycoplasma T.C. Rapid Detection System, GenProbe Inc., San Diego, CA, USA).

QUIN assay

QUIN was assayed by gas chromatography and electron-capture negative-chemical-ionisation mass spectrometry. Previous experiments had shown that the lower limit of detection was 40 nmol l⁻¹ (Heyes and Markey, 1988).

Experiment #1: Macrophage production of QUIN

0.5 ml of supernatant of an ADC isolate (clinically characterized as ADC stage 3 and virologically characterized as having 4⁺ macrophage tropism) (1.25×10^3 TCID₅₀ ml⁻¹) and 0.5 ml of [¹³C₆]-tryptophan AIMV medium were added to one set of macrophages; this was also performed with a non-ADC isolate (stage 0 and 1⁺ macrophage tropism) (1.25×10^3 TCID₅₀ ml⁻¹). 0.5 ml of the supernatant from the SUPT1 cells and 0.5 ml of [¹³C₆]-tryptophan labelled AIMV medium were added to another set of macrophages. Three 1ml aliquots of supernatant samples from the macrophages were taken at 24, 36, 48 and 60 h for assay of [¹³C₆]-QUIN. In earlier experiments, aliquots had been taken at 6 and 12 h but QUIN had not been detected. At each of the time points the number of viable cells in each of the wells was quantified after the supernatant had been removed for QUIN assay. Viability of cells was assessed by an ELISA reader after MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; thiazolyl blue; Sigma chemicals, St Louis, MD, USA) had been added. No evidence of cell death was found at any of the time points. To ensure that the isolates had infected the macrophages, *in situ* hybridization for HIV-1 mRNA was performed at 24 h: the HIV-1 gag protein was labelled with digoxigenin (DIG RNA Labelling Kit SP6/T7) and hybridized *in situ* with the HIV-1 RNA from the macrophages. The digoxigenin labelled RNA was then detected with an alkaline phosphatase conjugated antibody and visualised in the enzyme substrate.

Experiment #2: Relationship between macrophage production of QUIN and viral inoculum

The latter experiments were repeated with increasing inoculating dose (20, 40, 200, and 500 TCID₅₀ ml⁻¹) of a 3⁺ macrophage tropic isolate of HIV-1 from a patient with severe (stage 3) ADC and with a 1⁺ macrophage tropic isolate of HIV-1 from a patient without ADC.

Experiment #3: Relationship between macrophage production of QUIN and cellular factors

The experiments were then repeated using HIV-1 isolates that had been purified by centrifugation to

minimize the possibility of cellular factors such as cytokines inducing QUIN production. Supernatants from macrophages infected with an ADC (stage 3 4⁺ macrophage tropic) isolate and a non-ADC (stage 0 1⁺ macrophage tropic) isolate were each filtered through a millipore filter (0.22µ) and 20 ml were placed in a centrifuge underlaid with a 10 ml glycerol cushion (200 ml solution was made containing 20% glycerol (40 ml), 0.05M KCl (1.5g) and 1M TRIS-HCl (10 ml) pH 7.5). After centrifugation at 16,000 rpm for 2 h pellets were resuspended in 2 ml AIMV [¹³C₆]-tryptophan media which was then used to inoculate macrophages. The pellet was then assessed for the presence of viral peptide by immunoblotting and *in situ* hybridization for HIV-1 mRNA was used to assess productive infection after the pelleted virus samples had been inoculated onto the macrophages.

Experiment #4: Relationship between macrophage production of QUIN and degree of macrophage tropism

This was assessed by two methods: firstly by inoculating macrophages with isolates of HIV-1 taken from patients with and without ADC with differing degrees of macrophage tropism and secondly by using well characterized laboratory isolates of HIV-1.

QUIN production was assessed in macrophages that were infected with an HIV-1 isolate from a patient with ADC (stage 2) that was 1⁺ macrophage tropic and compared to an isolate that was 2⁺ macrophage tropic from a patient without ADC (stage 0). The viral inoculum for both isolates was 1×10^3 TCID₅₀ ml⁻¹. Second, QUIN production was assessed in macrophages that were infected with two different strains of HIV-1 that are known to have markedly different degrees of macrophage tropism: HIC-1_{SF2} which can only replicate to low levels in macrophages and HIV-1_{SF33} which replicates well in macrophages (York-Higgins *et al*, 1990). The same inoculum of 1.58×10^3 TCID₅₀ ml⁻¹ was used for each virus. *In situ* hybridization for HIV-1 mRNA was performed at 24 h for each of the isolates.

The same protocols for infection and the same ADC and non-ADC isolates that were used to inoculate macrophages were used for lymphocytes. Similarly, lymphocytes were infected with HIV-1_{SF2}, a known lymphotropic strain of HIV-1. [¹³C₆]-QUIN production by infected lymphocytes was assessed by three 1ml aliquots of supernatant samples from the lymphocytes taken at 24, 36, 48 and 60 h.

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