Journal of NeuroVirology, 7: 56–60, 2001
 ISSN 1355–0284/01 $12.00+.00

Short Communication

Quinolinic acid is produced by macrophages stimulated by platelet activating factor, Nef and Tat

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Activated macrophages produce quinolinic acid (QUIN), a neurotoxin, in several inflammatory brain diseases including AIDS dementia complex. We hypothesized that IL1-β, IL6, transforming growth factor (TGF-β_2) and platelet activating factor could increase macrophage QUIN production. And that the HIV-1 proteins Nef, Tat and gp41 may also increase synthesis of QUIN by macrophages. At 72 h there were significant increases in QUIN production in the cells stimulated with PAF (914 $\pm$ 50 nM) and Nef (2781 $\pm$ 162 nM), with somewhat less production by Tat stimulation (645 $\pm$ 240 nM). The increases in QUIN production approximated *in vitro* **concentrations of QUIN shown to be neurotoxic and correlated closely with indoleamine 2,3-dioxygenase induction. IL1-β, IL6, TGF-β_2 and gp41 stimulation produced no significant increase in QUIN production. These results suggest that some of the neurotoxicity of PAF, nef and tat may be mediated by QUIN.** *Journal of NeuroVirology* (2001) **7,** 56–60.

Keywords: HIV-1; ADC; kynurenine pathway

Tryptophan is catabolised by the kynurenine pathway (KP), the first enzyme of which is indoleamine 2,3-dioxygenase (IDO (ECI. 13.11.17) (Heyes *et al*, 1996; Stone, 1993). Cytokines such as interferon gamma (IFN-γ) and alpha (IFN-α) and to a lesser extent TNF-α can induce IDO (Heyes *et al*, 1996; Pemberton *et al*, 1997). The KP has several neurologically active metabolites such as quinolinic acid (QUIN) (Stone, 1993). QUIN is a neurotoxin that acts as an agonist at N-methyl-D-aspartate (NMDA) receptors raising intracellular calcium and leading to cell death (Stone, 1993). Elevated concentrations of QUIN are found in the cerebrospinal fluid (CSF) and brains of patients suffering from a variety of inflammatory brain diseases including the AIDS dementia complex (ADC) (Heyes *et al*, 1996). QUIN's neurotoxicity has been demonstrated in cell culture systems and animal models (Kerr *et al*, 1995; 1998). QUIN causes dendritic injury at low concentrations and excitotoxic cell death at higher concentrations. These changes are consistent with the neuropathology of ADC (Kerr *et al*, 1998; 1997; Masliah *et al*, 1997).

The severity of ADC correlates best with the number of activated cells of monocytic lineage within the brain rather than the level of productive HIV infection (Glass *et al*, 1995). The CSF of ADC patients has been found to have a pro-inflammatory cytokine profile with particular elevation in interleukin 1β (IL1-β), interleukin 6 (IL6). Because of the relationship between QUIN and inflammation, we hypothesised that these pro-inflammatory cytokines would lead to increased macrophage production of QUIN. Moreover, the activated monocyte lineage cells produce a number of neurotoxins among which are platelet activating factor (PAF) and the viral proteins gp120, Nef and Tat (Nath and Geiger, 1998). The precise mechanism of their neurotoxicity is still not completely understood. Previously we have shown that gp120 does not induce macrophage QUIN production (Pemberton *et al*, 1997). We hypothesised that PAF, gp41, Nef and Tat

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Received 16 June 2000; revised 5 August 2000; accepted 23 August 2000

would induce human macrophages to produce significant amounts of QUIN. We also explored the role of TGF-β_2 in QUIN production as it is present in the brains of patients with ADC in an inverse proportion to TNF-α (Achim *et al*, 1996) and it can reduce the activity and expression of IDO in human fibroblasts after stimulation with IFN-γ (Yuan *et al*, 1998).

Stimulation of macrophages with the cytokines IL1-β, IL6 and TGF-β_2 alone induced no significant increase in QUIN production (Table 1) nor did the addition of TGF-β_2 reduce the level of QUIN produced by IFN-γ stimulated cells. At the concentrations of 0.05 and 0.5 ng/ml of PAF, the macrophages produced low but significant amounts of QUIN in comparison to the negative control. PAF at 50 ng/ml seemed to have a toxic effect on the macrophages as there was a high occurrence of dead cells in the medium. PAF at 5 ng/ml produced the most QUIN at 914 ± 50 nM with a *P* value of <0.0001 (Table 2) compared to the negative control but significantly less than the positive control ($P=0.02$).

Stimulation of macrophages with the higher concentration of Nef (1 μg/ml) produced high levels of QUIN at 72 h. The mean value for QUIN production was 2781 ± 162 nM, which was significantly higher than the negative controls ($P<0.0001$) but significantly lower when compared with the positive control ($P=0.05$).

Treatment of macrophages with the higher concentration of Tat (100 nM or 1.4 μg/ml) resulted in a small increase in QUIN concentration at 48 and 72 h. The mean concentration of QUIN produced at 72 h was 645 ± 240 nM, was significantly higher then the negative control ($P=0.01$), though it was significantly lower then the positive control ($P=0.02$).

Treatment of macrophages with both concentrations (0.2 and 2 μg/ml) of gp41 did not result in a significant increase in QUIN production. The results were not significantly higher than the mean of the negative control.

An accurate correlation has been observed between QUIN production by macrophages stimulated by IFN-γ, PAF, Tat and Nef and induction of

Table 1 Statistical comparison of QUIN production by human macrophages stimulated with IFN-γ IL1-β, IL6 and TGF-β_2 alone and in combination with IFN-γ

Treatment	*Mean [QUIN]* ± *s.e.* (nM) *at 72 h*[a]	*Negative control* P-*value*[b]	*Positive control* P-*value*[b]
Negative control	85 ± 48	*	<0.0001
IFN-γ (positive control)	8900 ± 922	<0.0001	*
IL1-β	180 ± 21	NS	<0.0001
IL6	152 ± 70	NS	<0.0001
TGF-β_2	112 ± 49	NS	<0.0001
TGF-β_2 + IFN-γ	8301 ± 126	0.0003	NS

[a]Mean QUIN production (nM) ± 1 s.e. at 72 h. [b][QUIN] were compared among the different groups, and *P* values were generated. [c]NS, *P* values were not statistically significant.

Table 2 Statistical comparison of QUIN production by human macrophages stimulated with IFN-γ, PAF, Tat, gp41 and Nef

Treatment	*Mean [QUIN]* ± *s.e.* (nM) *at 72 h*[a]	*Negative control* P-*value*[b]	*Positive control* P-*value*[b]
Negative control	155 ± 44	*	0.0002
IFN-γ (positive control)	11004 ± 2050	0.0002	*
PAF 50 ng/ml	169 ± 21	NS[c]	0.01
PAF 5 ng/ml	914 ± 50	<0.0001	0.02
PAF 0.5 ng/ml	379 ± 18	0.02	0.02
PAF 0.05 ng/ml	434 ± 186	0.02	0.02
Tat 100 nM	645 ± 240	0.01	0.02
Tat 10 nM	114 ± 33	NS[c]	0.01
Tat 1 nM	52 ± 12	NS[c]	0.01
Tat 100 nM (heat inact)	152 ± 70	NS[c]	0.04
gp41 2 μg/ml	105 ± 32	NS[c]	0.01
gp41 0.2 μg/ml	91 ± 7	NS[c]	0.01
Nef 1 μg/ml	2781 ± 162	<0.0001	0.05
Nef 0.1 μg/ml	182 ± 7	NS[c]	0.01
Nef 0.01 μg/ml	219 ± 18	NS[c]	0.01

[a]Mean QUIN production (nM) ± 1 s.e. at 72 h. [b][QUIN] were compared among the different groups, and *P* values were generated. [c]NS, *P* values were not statistically significant.

IDO mRNA expression (Figure 1). No RT – PCR signal was detected in macrophages cultured with basal AIM-V medium (Negative control), and a very strong signal was observed with IFN-γ stimulated macrophages (Positive control). The PCR results for gp41, IL1-β, IL6 and TGF-β_2 correlated with the lack of QUIN production in that no RT – PCR signal was detected, hence we did not include these results in Figure 1.

In accordance with QUIN quantification results, PAF 0.05, 0.5 and 50 ng/ml led to a weak induction of expression of IDO with PAF 5 ng/ml inducing a much higher response. Tat induced IDO expression in a dose dependent manner. Tat 100 nM heat inactivated (100 nM HI) was still able to induce IDO expression but to a lesser extent than TAT 100 nM. Nef 0.01 and 0.1 μg/ml had a weak effect on IDO induction whereas Nef 1 μg/ml led to a high level of expression of the enzyme (2.6-fold $<$to positive control).

We have shown that *in vitro* stimulation of macrophages with PAF and the HIV-1 viral proteins Tat and Nef at pathophysiologically appropriate concentrations resulted in significantly increased QUIN production by these cells. The level of QUIN produced was equal to or above the levels (a) found to cause neuropathological damage in *in vitro* systems (Kerr *et al*, 1998; Whetsell and Schwarcz, 1989 and (b) found in the CSF of demented patients (Heyes *et al*, 1991a,b). The cytokines IL1-β, IL6 and the viral protein gp41 did not cause a significant increase in QUIN production nor did TGF-β_2 reduce the stimulatory effect of IFN-γ.

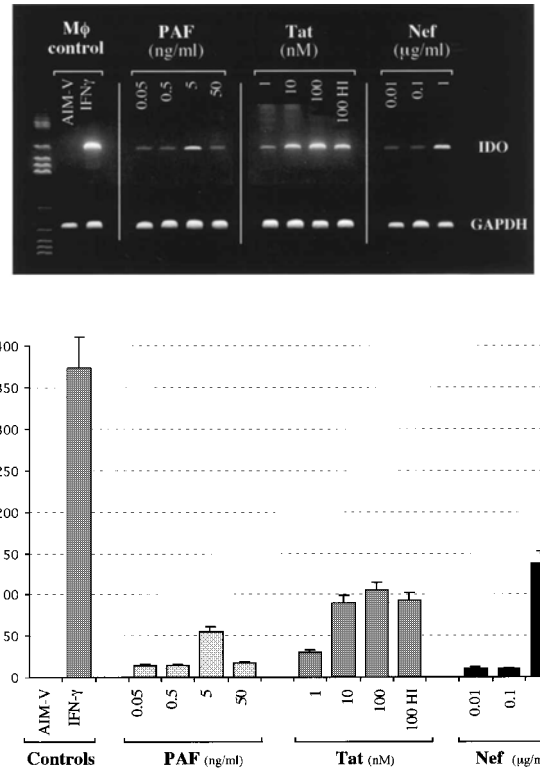

Figure 1 (top) Photograph of ethiduim bromide stained gel showing RT-PCR for IDO mRNA expression, 72 h after treatment with PAF, Tat and Nef. Human primary macrophages (MΦ) stimulated with IFNγ were used as a positive control for IDO expression. (bottom) Histogram showing image analysis intensity ratios of IDO mRNA expressed relative to GAPDH mRNA.

Nef, Tat and PAF are all considered neurotoxins in various models of ADC pathogenesis. However their modes of neurotoxicity are not well described. Nef transcripts are expressed in latently infected astrocytes which then preferentially produce rev and nef protein (Ranki *et al*, 1995). Nef expression in astrocytes may lead to a decrease in their normal detoxifying function (Saito *et al*, 1994). Additionally, Nef may be able to block the normal proliferative response to platelet derived growth factor and nerve growth factor in fibroblastic cell lines (De and Marsh, 1994). Nef has been found to have structural similarities to scorpion neurotoxin thus offering one mode for its neurotoxicity (Werner *et al*, 1991).

Tat has been shown in other studies to be neurotoxic when injected into the brains of rats (Magnuson *et al*, 1995; Brana *et al*, 1999) and when added to human cerebrocortical cultures with QUIN (Chao *et al*, 2000). But it is not certain whether Tat neurotoxicity is direct or whether a neurotoxic cascade is induced. Tats neurotoxicity can be completely blocked using a glutamate receptor antagonist such as memantine (Nath *et al*, 2000).

PAF is recognised as an important neurotoxin in the progression of ADC. It is a lipid inflammatory mediator shown to cause neuronal death and is inducible by IFN-γ (Gelbard *et al*, 1994; Schifitto *et al*, 1999). It can upregulate TNF-α synthesis by HIV infected monocytes and like TNF-α, induce dose dependent apoptosis. NMDA receptor channel antagonists ameliorate PAF induced neurotoxicity (Perry *et al*, 1998).

Our data suggest that in addition to the above mentioned mechanisms, QUIN mediates at least part of the neurotoxicity of PAF, Nef and Tat. Because PAF neurotoxicity is related to NMDA receptor activation, we suggest that PAF's neurotoxicity is mediated dominantly if not exclusively by QUIN. Conversely, Nef and Tat have a number of potential mechanisms of neurotoxicity, one of which could be mediated by QUIN.

The mechanisms by which PAF, Nef and Tat induce QUIN production by macrophages are unclear. However, it is possible that they could directly effect the kynurenine pathway by inducing IDO, but until now few inducers of this enzyme have been described. Another explanation is that PAF, Nef and Tat may have an indirect effect mediated by IFN-γ synthesis, which then induces IDO. Alternatively, they could cause the induction

of other intermediates, which then may induce the KP. At present, though there are no data to make any of these possibilities more likely.

Our data also have more general implications for the control of QUIN production in other disease states. Currently, IFN-γ, IFN-α and to a lesser extent TNF-α are considered the only factors capable of increasing QUIN production by macrophages (Pemberton *et al*, 1997). Our data broaden this to include another inflammatory mediator PAF. More importantly, these results show that QUIN is not simply a marker of a proinflammatory milieu as IL1-β and IL6 did not affect QUIN production. Moreover, the fact that Nef and Tat could induce QUIN productions suggests that other non-HIV viral proteins may also be able to do so.

These data go further to underscore the importance of QUIN in the model of ADC pathogenesis and in other inflammatory or virally mediated brain diseases. Inhibitors of the kynurenine pathway would be a future direction for clinical trials for the control of ADC.

When preparing the macrophages, all cell culture media and additives were obtained from Life Technologies (Gaithersburg, USA) unless otherwise stated. Macrophages were isolated from 120 ml peripheral blood of HIV-1 seronegative volunteers using the standard Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density separation method. The cell pellet was resuspended in macrophage feeding medium consisting of RMPI-1640 supplemented with 10% heat-inactivated donor human serum, 2 mM glutamine, 200 U/ml penicillin G, 200 μg/ml streptomycin sulphate and 5 mM HEPES buffer, to give a density of 5×10^6 cells/ml. One ml of cell suspension was seeded into the eight centre wells of 24-well tissue culture plates (Corning, Corning NY, USA). The surrounding wells were filled with PBS, to minimise changes in volume due to evaporation in the incubator, and kept at 37°C in a humidified atmosphere with 5% CO_2. After 2 h, each well was washed twice with PBS to remove non-adherent cells then fed. After 3 days, the medium was changed to 1 ml of equal quantities of human serum supplemented RPMI and a serum free medium, AIM-V. At 7 days, the serum component of the medium was eliminated completely to reduce the endogenous QUIN level and the cells were maintained in basal AIM-V medium.

Recombinant IL1-β, IL6, IFN-γ, TGF-β2 and PAF were obtained from Roche (Roche Diagnostics, Branchburg, NJ, USA). All cytokines were diluted in AIM-V medium to the following concentrations: IFN-γ, IL1-β, IL6 (100 IU/ml), TGF-β2 (10 ng/ml), PAF (0.05, 0.5, 5 and 50 ng/ml). The Tat protein was kindly provided by Dr Avi Nath (Department of Neurology, University of Kentucky, Lexington, USA), the Nef protein by Prof Azad (Azad *et al*, 1994) (Biomolecular Research Institute, Parkville, Victoria, Australia). All viral proteins were diluted in AIM-V medium to the following concentrations: Tat (1000, 100, 10 and 1 nM), heat inactivated Tat (100 nM), Nef (0.01, 0.1 and 1 μg/ml), gp41 (Immunotech) (0.2 and 2 μg/ml). The concentrations of the latter cytokines and viral proteins are similar to those used by other investigators and approximate those seen in pathophysiological conditions,

Nine day-old macrophages were treated with 1 ml of one of the following cytokines or viral proteins: IFN-γ (100 IU/ml), IL1-β (100 IU/ml), IL6 (100 IU/ml), TGF-β2 (10 ng/ml), PAF (0.05, 0.5, 5 and 50 ng/ml), Tat (1000, 100, 10 and 1 nM), heat inactivated Tat (100 nM), Nef (0.01, 0.1 and 1 μg/ml), gp41 (0.2 and 2 μg/ml). There was also a treatment group with TGF-β2 (10 ng/ml) and IFN-γ (100 IU/ml) added simultaneously. The cells were then incubated at 37°C in an environment of 5% CO_2. Triplicate 120 μl samples were taken at 24, 48 and 72 h. Unstimulated macrophages in AIM-V medium alone were used as a negative control. Macrophages stimulated with IFN-γ (100 IU/ml) were used as a positive control. PAF is grouped with the viral proteins in Table 1 as it was assayed using the same controls as the proteins. Tat heat inactivated at 100°C for 10 min was used as a control for endotoxin contamination. The Nef and Tat proteins were tested for endotoxin contamination using the Limulus lysate-Pyrotell assay (Associates of Cape Cod Inc., Falmouth, MA).

One hundred microlitres of culture supernatant was analysed for QUIN by electron-capture negative ionisation gas chromatography/mass spectrometry (GC/ECNI-MS) as described by Pemberton *et al* (1997). RT – PCR detection of IDO mRNA expression. The complete method and primers sequences have been described by Guillemin *et al* (1999).

Mean values and standard errors were calculated for each treatment at all time points, and the results were plotted on a histogram. Unpaired *t* tests were performed on the results obtained at 72 h. *P* values were generated comparing QUIN production from the various treatments to the negative and positive controls. A *P* value of <0.05 was regarded as statistically significant.

Acknowledgements

This work was supported by a grant from the National Health and Medical Research Council and an infrastructure grant from the New South Wales Health Department. Special thanks to Prof Azad for generously providing us with our nef protein.

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