

# Toxicity of $\text{TNF}\alpha$ and platelet activating factor for human NT2N neurons: a tissue culture model for human immunodeficiency virus dementia

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A significant proportion of HIV-1 infected individuals develop a symptom complex consisting of dementia and motor deficits termed HIV Dementia (HIVD) or the AIDS Dementia complex (ADC). The pathophysiology of this neurologic complication is unclear, but neuronal injury and death may occur as a direct result of the release of cytokines from HIV-1 infected microglial cells (Everall *et al*, 1991). To evaluate the utility of a human neuronal cell line, NT2N, for studies of HIV-related neuronal cytotoxicity, we studied cellular viability after exposure to HIV-1 gp120, tumor necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ), platelet activating factor (PAF), interleukin 1 beta ( $\text{IL-1}\beta$ ), and interferon gamma ( $\text{IFN}\gamma$ ), all of which have been implicated in previous publications as having a role in HIVD (Brenneman *et al*, 1988; Dreyer *et al*, 1990; Merrill *et al*, 1992; Gelbard *et al*, 1993). Neither gp120 nor the cytokines  $\text{IL-1}\beta$  and  $\text{IFN}\gamma$  resulted in significant NT2N cell death. However,  $\text{TNF}\alpha$  and PAF were highly neurotoxic in this assay. Pentoxifylline, which inhibits the effects of  $\text{TNF}\alpha$ , had a significant protective effect. This system provides an excellent substrate for the evaluation of neurotoxicity and for the development of pharmacologic agents that may be useful in HIV dementia.

**Keywords:** HIV-1; neurotoxicity; apoptosis;  $\text{TNF}\alpha$ ; PAF; gp120

## Introduction

Many human individuals infected with the type-1 Human Immunodeficiency Virus (HIV-1) develop a neurologic syndrome characterized by cognitive and motor deficits termed HIV dementia (HIVD) or the AIDS Dementia complex (reviewed by Berger and Kaderman, 1995). These clinical abnormalities are frequently, though not always, accompanied by central nervous system (CNS) neuropathological changes including neuronal loss, myelin pallor, dendritic vacuolation, reactive astrocytosis, and infiltration by microglia and multinucleated giant cells (Lang *et al*, 1989; Glass *et al*, 1993; reviewed by Wiley and Achim, 1995).

Although HIVD is thought to be due to HIV-1 infection itself, and not the result of a secondary infection with an opportunistic pathogen, its pathophysiology remains unclear. In spite of some evidence of neuronal dropout, neuronal infection

by HIV-1 has not been detected with conventional immunohistochemical or *in situ* hybridization methods, although recent *in situ* PCR experiments have demonstrated HIV-1 sequences in the neurons of some patients with advanced HIVD (Nuovo *et al*, 1994; Bagasra *et al*, personal communication). Nevertheless, while it is possible that a restricted infection of neurons could interfere with the complex physiology of these highly specialized cells and perhaps even be responsible for some cell death, any explanation of the pathogenesis of HIVD must account for the role of microglia, the CNS cell type that has been most consistently found to be productively infected with HIV-1. Thus, one proposed mechanism for neuronal injury and death is that it occurs as a result of the release of cytokines from infected microglial cells (Everall *et al*, 1991; Epstein and Gendelman, 1993). Astrocytes, which may also show evidence of a restricted infection with HIV-1 (Blumberg *et al*, 1993), could also interact with microglia to either contribute neurotoxins from their repertoire of potential cytokines or to stimulate microglia to increase the production of neurotoxins.

Several investigators have shown that macrophages exposed to HIV-1, or even its envelope protein, gp120, will produce TNF $\alpha$ , IFN $\gamma$ , IL-1 $\beta$ , IL-6, platelet activating factor (PAF), and arachidonic acid metabolites (Wahl *et al*, 1989; Merrill *et al*, 1992; Genis *et al*, 1992; Tyor *et al*, 1992; Epstein and Gendelman, 1993; Gelbard *et al*, 1993; Yeung *et al*, 1995; Brew *et al*, 1995). In addition unidentified soluble factors from HIV-infected macrophages have been shown to cause neuronal death in cultured human brains (Pulliam, 1995; Gelbard *et al*, 1993, 1994; Genis *et al*, 1992).

Of all the potential neurotoxins that could be released by microglia, TNF $\alpha$ , quinolinic acid and PAF have received the widest attention. Steady-state levels of TNF $\alpha$  mRNA are higher in the subcortical regions of the CNS in patients with HIVD than in HIV infected patients without CNS involvement (Wesselingh *et al*, 1993). Furthermore, TNF $\alpha$  has also been identified in the cerebrospinal fluid (CSF) of HIV infected patients (Tyor *et al*, 1992). Although it has not received as much emphasis, PAF was also shown to be elevated in HIV infection (Gelbard *et al*, 1994). Collectively, these studies have suggested that neuronal death is due to complex cellular interactions that involve the release of neurotoxins – including cytokines and arachidonic acid metabolites – by microglia and astrocytes infected by HIV-1 or activated by gp120, as well as due to alterations of astrocytic function.

With few exceptions (Pulliam, 1995; Gelbard *et al*, 1994), studies of HIV-1 associated neuronal injury have depended on cultured neurons isolated from rat brain. Although the pharmacologic mechanisms that relate to cell death may apply to several mammalian species, the preponderance of studies that use rodent brain have left many unanswered questions, not the least of which is the physiologic relevance of gp120 binding to cells from species that do not serve as hosts for the virus. Additionally, rodent or human primary brain cultures contain several cell types, making it harder to assess the contribution of individual substances on each cell type. We have adapted a system using human cells in order to clarify some of these issues.

NT2N neurons are differentiated from human teratocarcinoma cells during a four week course of treatment with retinoic acid (Pleasure *et al*, 1992). The differentiated cells resemble mature post-mitotic human neurons in morphology and function, and they express neurotransmitter enzymes (Llanes *et al*, 1995), neuronal markers including neurofilaments, growth-associated protein 43 (Pleasure *et al*, 1992) and NMDA and non-NMDA glutamate receptors (Younkin *et al*, 1993). NT2N cells can be infected by some HIV-1 strains (D Kolson, unpublished results). We report initial experiments that demonstrate the potential use of these cells for studies of HIVD.

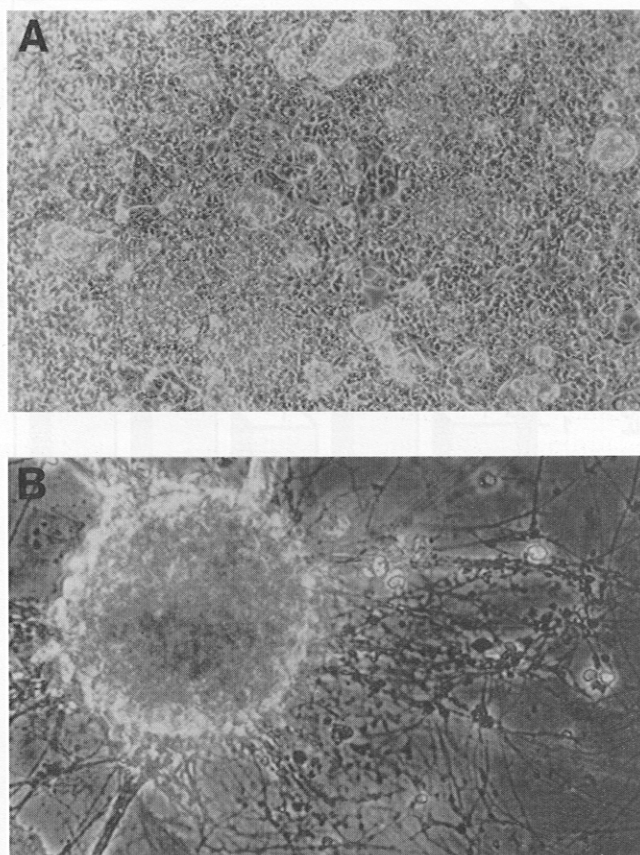
## Results

### *Differentiated NT2N*

Using a protocol described by Pleasure *et al* (1992) NT2 cells were differentiated into a neuronal phenotype (NT2N) over several weeks. Towards the end of the differentiation period the NT2N cells showed neuronal morphology, including the development of neurites (see Figure, 1). These morphological changes have been associated with the development of neuronal markers like neurofilament proteins, NMDA receptors (Younkin *et al*, 1993; Pleasure & Lee, 1993), and neurotransmitter enzymes (Llanes *et al*, 1995). Of particular relevance to these experiments, NMDA receptors are present in greatest amount between 4–6 weeks after terminal differentiation.

### *gp120 effects on NT2N*

The HIV-1 surface glycoprotein gp120 has been implicated in neuronal toxicity in several cultured and *in vivo* rodent models (Brenneman *et al*, 1988; Dreyer *et al*, 1990; Lipton *et al*, 1991). To evaluate



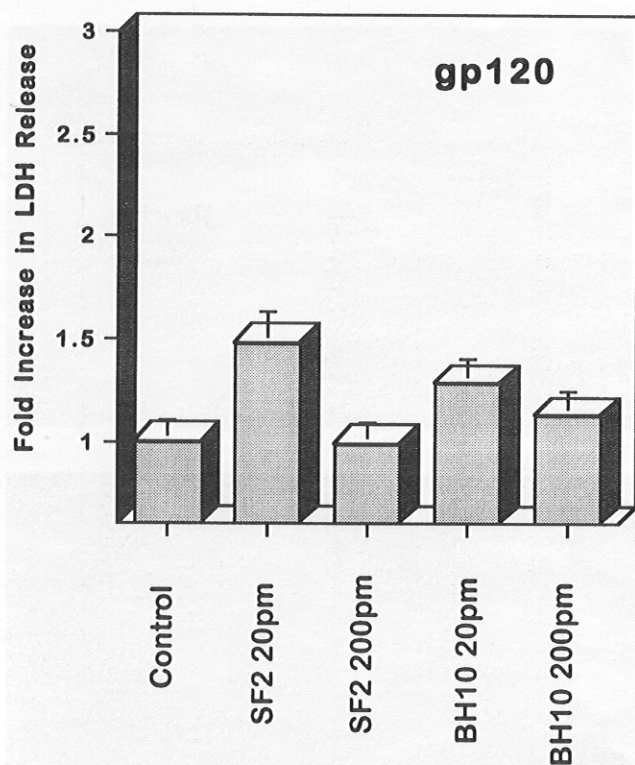
**Figure 1** NT2 cells in culture (A) NT2 cells in the process of differentiation (2 weeks after commencing treatment with retinoic acid) under phase contrast microscopy. The cells exhibit morphology typical for the undifferentiated teratocarcinoma cells. (B) Terminally differentiated NT2 (NT2N) cells demonstrating typical cellular aggregates as well as the presence of neuritic processes.



its direct neuronal cytotoxicity, we exposed NT2N cells to recombinant gp120 (rgp120) preparations cloned from two HIV strains, HIV-1<sub>SF2</sub> and HIV-1<sub>IIIB</sub> (BH10). Neuronal death was measured with the LDH release assay (Choi *et al*, 1987; Choi, 1988). As shown in Figure 2, at 27 h after exposure there was a minimal rise in the amount of LDH released with 20 pM of either rgp120 preparation when compared to cells treated with buffer alone. Neither increasing the concentration of rgp120 to 200 pM (Figure 2) nor the length of exposure to 72 h (not shown) resulted in increased toxic effects. These results indicated that rgp120 alone is not toxic to NT2N neurons, and suggested that the effects seen in other systems are either due to the presence of other neural cells in the culture, or are very specific to the particular rgp120 preparation.

#### Neuronal toxicity of microglial and astrocytic cytokines

We then looked at the effects of several cytokines in the same assay. IL-1 $\beta$ , IFN $\gamma$  and TNF $\alpha$  have all been implicated as potential neuronotoxins in HIVD



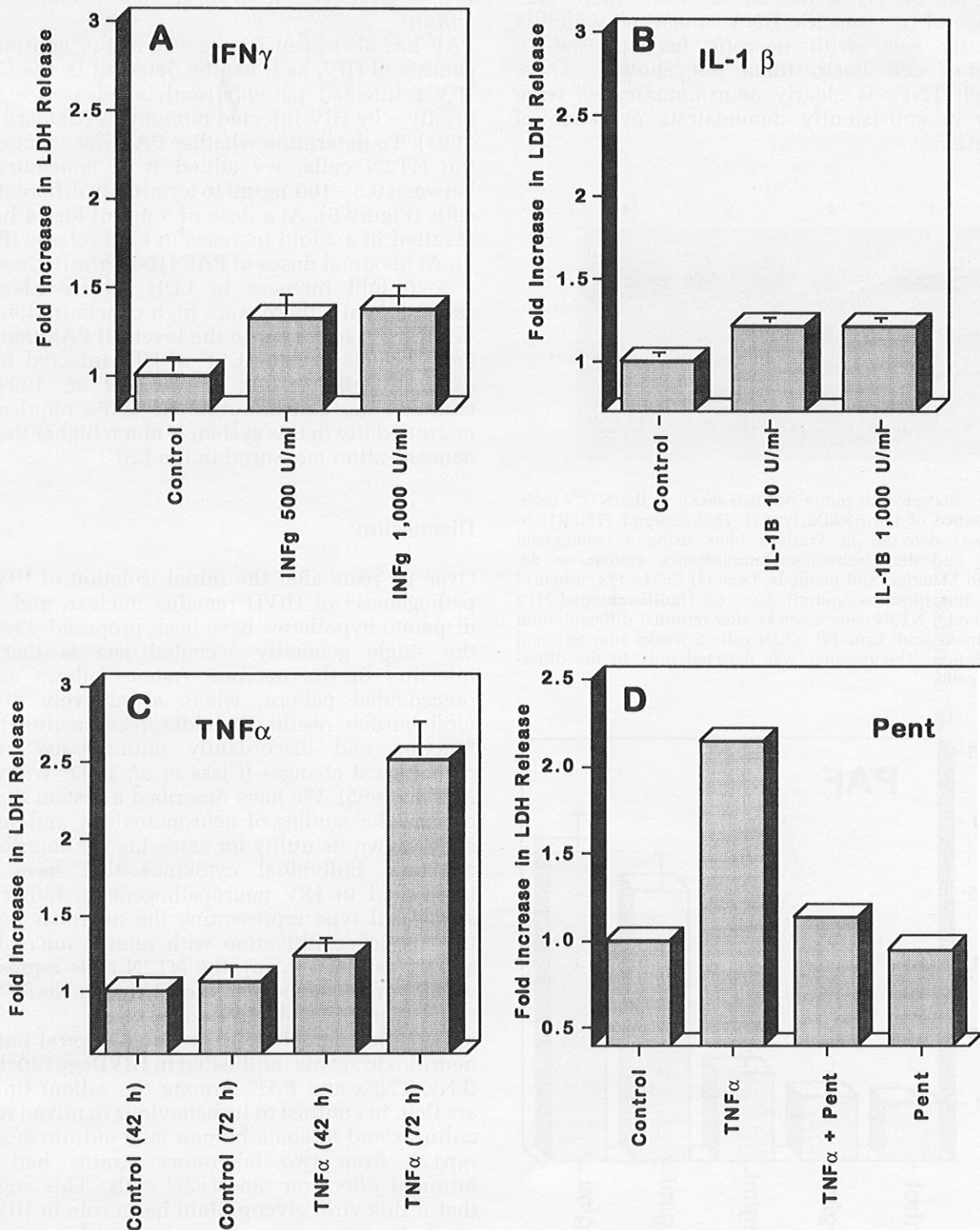
**Figure 2** Effect of gp120 on NT2N cells. Terminally differentiated NT2 cells (NT2N) were exposed to gp120 from either HIV-1<sub>SF2</sub> or HIV-1<sub>IIIB</sub> (BH10) at two concentrations, 20 pM or 200 pM and the supernatants were removed 27 h later. LDH release in each well was measured in quadruplicate as indicated in the Materials and methods section, and the average of three replicate wells containing equivalent numbers of cells is represented as the ratio of LDH in the experimental wells to that in the control (serum free medium) wells. Cells exposed to 20 pM of HIV-1<sub>SF2</sub> gp120 had an LDH release level 1.5 $\times$  background, but this effect was not dose-dependent and was thus not considered significant.

(Genis *et al*, 1992; Masliah *et al*, 1994), and IL-1 $\beta$  is released from infected monocytes (Wahl *et al*, 1989). As shown in Figure 3A, treatment with IFN $\gamma$  for up to 72 h at a concentration of 500 U/ml resulted in a level of LDH release that was somewhat above background, but the level did not increase in parallel with increases in the concentration of cytokine. Similarly, treatment with 1 000 U/ml of IL-1 $\beta$  for 48 h (Figure 3B) had minimal effects on the release of LDH from the differentiated NT2N. Given the modest rises in LDH in comparison to those produced by other cytokines (Figures 3C and 5), we did not consider these significant, and concluded that neither of these cytokines results in neuronal death in this system.

TNF $\alpha$  has been implicated in HIVD by studies demonstrating an increase in its steady-state mRNA levels (Wesselingh *et al*, 1993), as well as by experiments documenting its release from infected or gp120-treated macrophages or microglia (Merrill *et al*, 1992; Nokta *et al*, 1995; Wilt *et al*, 1995). As shown in Figure 3C, treatment of NT2N with 50 ng/ml TNF $\alpha$  for 72 h increased the level of LDH release 2.5-fold in comparison to control cells treated with buffer only. Treatment at concentrations of up to 500 ng/ml for 66 h also resulted in 2.5–3 fold increase in LDH release over background (data not shown). However, because the requirement for serum-free medium for the LDH release assay leads to decreased survival of the NT2N cells, there was significant LDH release in the untreated cells during the longer exposure times. To confirm the specificity of the effects noted with TNF $\alpha$ , we used pentoxifylline, a known inhibitor of its toxicity. As shown in Figure 3D, at a concentration of 30  $\mu$ g/ml pentoxifylline completely abolished the cytotoxic effects of a large dose of TNF $\alpha$  on the NT2N. These results were confirmed in separate experiments using the MTT assay as an index of survival (data not shown).

The effect of TNF $\alpha$  in most cell types has been shown to involve activation of the TNF receptor type 1 (TNFR1). To ascertain this receptor was present in the NT2N cells, we performed a Western analysis using a monoclonal antibody (see Materials and methods). As shown in Figure 4, the 55 kDa molecule was easily detected in differentiated NT2N cells after either 4 or 5 weeks of treatment with retinoic acid. TNFR1 was not present in the undifferentiated cells (NT2 or uNT2).

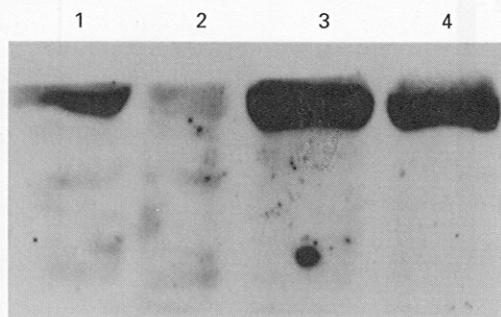
Activation of TNFR1 triggers a series of cellular pathways that result in cell death; in certain cell types this death is due to apoptosis (programmed cell death). To determine whether the toxicity observed following TNF $\alpha$  treatment in the NT2N cells was due to apoptosis, we harvested cellular DNA after exposure to TNF $\alpha$ , and analyzed it for fragmentation. Fragmentation, as assayed by several different methods, is evidence that apoptosis is responsible for cell death. Following treatment with



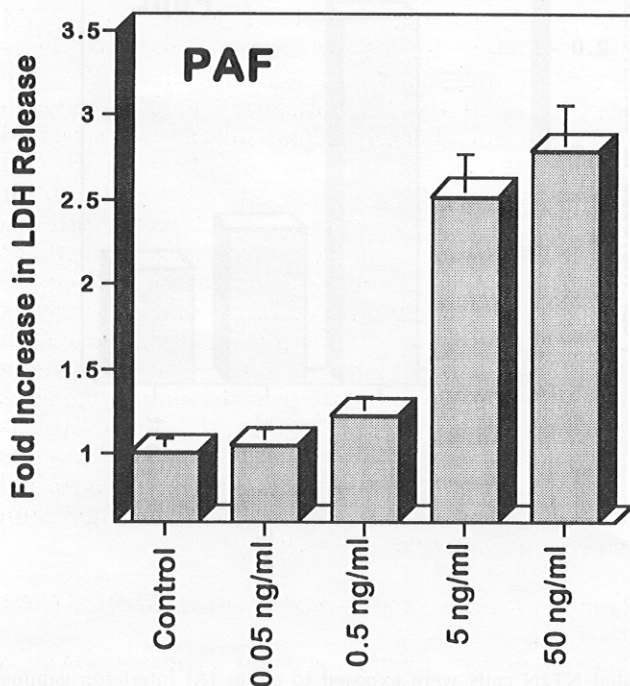
**Figure 3** Effects of Cytokines on NT2N cells. Terminally differentiated NT2N cells were exposed to either (A) interferon gamma (IFN $\gamma$ ), (B) interleukin 1 $\beta$  (IL-1 $\beta$ ) or (C) TNF $\alpha$ . Seventy-two hours after exposure (for IFN $\gamma$  and TNF $\alpha$ ) or 48 h after exposure (for IL-1 $\beta$ ) the medium from each well was removed and assayed for LDH in quadruplicate, as indicated in Materials and methods. The results are expressed as a ratio of the average of three experimental wells containing equivalent numbers of cells for each condition over the average of three control wells (maintained in serum-free medium for the same period). Cells exposed to 50 ng/ml TNF $\alpha$  for 72 h had a significant increase in LDH release when compared with control cells treated with serum-free medium for the same period of time (see text). In (D) a vast excess of TNF $\alpha$  (400U or 2  $\mu$ g/ml) was used, but the cells were simultaneously exposed to pentoxifylline, an inhibitor of TNF activity. Pentoxifylline almost completely abolished the effects of TNF $\alpha$  noted at this dosage.



500 ng/ml of TNF $\alpha$  for 26 or 48 h, there was evidence of non-specific DNA 'smearing' which is frequently seen with necrotic (as opposed to apoptotic) cell death (data not shown). Thus, although TNF $\alpha$  is clearly neuronotoxic, we were unable to consistently demonstrate evidence of apoptosis.



**Figure 4** Receptor for tumor necrosis factor in the NT2N cells. The presence of the 55 kDa type 1 TNF receptor (TNFR1) in NT2N was detected in Western blots using a commercial antibody and the electrochemoluminescence system, as described in Materials and methods. Lane (1) CEM  $\times$  174, a hybrid B/T cell line (positive control); Lane (2) Undifferentiated NT2 cells; Lane (3) NT2N cells 4 weeks after terminal differentiation with retinoic acid; Lane (4) NT2N cells 5 weeks after terminal differentiation. The receptor was detected only in the differentiated cells.



**Figure 5** Effects of platelet activating factor (PAF) on NT2N cells. Terminally differentiated NT2N cells were exposed to PAF at several concentrations for 24 h and LDH release measured as described in Materials and methods. The average of triplicate wells is reported as fold increase over the LDH release in control wells (serum-free medium only).

### PAF is directly toxic to NT2N neurons alone in culture

PAF has also been implicated in the neuropathogenesis of HIV, as it can be detected in the CSF of HIV-1 infected patients, and is released – albeit briefly – by HIV-infected monocytes (Gelbard *et al*, 1994). To determine whether PAF was cytotoxic to the NT2N cells, we added it at concentrations between 0.5–100 ng/ml to terminally differentiated cells (Figure 5). At a dose of 5 ng/ml for 24 h, PAF resulted in a 2 fold increase in LDH release (Figure 5). At maximal doses of PAF (100 ng/ml), there was a 5–6 fold increase in LDH release (data not shown). While these were high concentrations, the lower ones are closer to the levels of PAF that have been detected in the CSF of HIV infected individuals (22–853 pg/ml; Gelbard *et al*, 1994). In contrast, the concentration of TNF $\alpha$  required for neurotoxicity in this system is much higher than the concentration measured in the CSF.

## Discussion

Over 10 years after the initial isolation of HIV, the pathogenesis of HIVD remains unclear, and many disparate hypotheses have been proposed. Probably the single generally accepted fact is that HIV infection of the nervous system follows an unprecedented pattern, where a relatively discrete viral burden results in widespread neuronal dysfunction and discordantly unimpressive neuropathological changes (Glass *et al*, 1993; Wiley and Achim, 1995). We have described a system that can be used for studies of neuronotoxicity and specifically shown its utility for assessing the contribution of some individual cytokines that have been implicated in HIV neuropathogenesis. Either as a single cell type representing the neuronal population, or in cocultivation with human microglia or astrocytes (not shown), the NT2N cells represent a unique *in vitro* model of the CNS suitable for these types of pharmacologic studies.

With this model we have tested several putative neurotoxic agents implicated in HIVD: gp120, IL-1 $\beta$ , IFN $\gamma$ , TNF $\alpha$  and PAF. Among the salient findings are that, in contrast to its behaviour in mixed rodent cultures and in some human fetal culture systems, rgp120 from two laboratory strains had only minimal effect on the NT2N cells. This suggests that if this viral glycoprotein has a role in HIVD, it must function in conjunction with astrocytes, microglia, and possibly oligodendrocytes, as has been previously hypothesized (Genis *et al*, 1992; Nottet *et al*, 1995). Alternatively, rgp120 toxicity may be highly dependent on the individual glycoprotein preparation or viral strain, or on the presence of specific neuronal sub-populations that are not represented in the NT2N system. Although rgp120 is known to bind to human astroglia (Ma *et*

*al*, 1994), to our knowledge specific binding to rodent culture systems has only been demonstrated for oligodendrocytes (Kimura-Korda *et al*, 1994). Furthermore, most or perhaps all of the experiments have been performed with rgp120 from laboratory strains, which are now recognized to be poorly representative of the HIV population present in infected individuals. Our studies would suggest that further work in this area should concentrate on human neuronal cells.

Neither IL-1 $\beta$  nor IFN $\gamma$  treatment killed these neuronal cells. In contrast, high doses of TNF $\alpha$  had an effect on LDH release that could be inhibited with pentoxifylline. As expected from these results, NT2N human neurons expressed abundant levels of TNFR1, even though it was not expressed in the undifferentiated NT2 cells. TNF $\alpha$  treatment results in activation of the NF- $\kappa$ B family of transcription factors; one of the results of this activation is the upregulation of some of the genes that have been implicated in apoptotic cell death. In fact, in one study, TNF $\alpha$  led to apoptosis in differentiated SK-N-MC cells (Talley *et al*, 1995). However, TNF $\alpha$  induces cell death by both apoptosis and necrosis depending on the cell type, and we could not detect any evidence that death of the NT2N cells was apoptotic. Furthermore, in view of its frequent implication as a mediator or neuronal dropout in HIVD, we were surprised at the levels of TNF $\alpha$  that were required to mediate cell death, which were much higher than have been measured in the CSF of HIV infected patients (Tyor *et al*, 1993). As these cells express high levels of the proto-oncogene *bcl-2* at points during their culture (Pekosz and Westmoreland, unpublished) the high levels of TNF $\alpha$  required to demonstrate toxicity are consistent with the findings of Talley *et al* (1995), who demonstrated an inverse correlation between *bcl-2* expression and toxicity mediated by TNF $\alpha$ . Unless the response of the NT2N cells is not representative of that of neuronal cells in general, one would have to postulate that in HIV infected individuals there are very high local levels of this cytokine. Alternatively, TNF $\alpha$  may be inducing its own synthesis, and its toxic effects due to intracellular concentrations. We were unable to neutralize the TNF $\alpha$  effect with antibodies, but we attributed this result to the high levels of the cytokine that are necessary to induce neuronal death.

Another cytokine recently implicated in HIVD is PAF; patients with severe HIVD (ADC stage 3) had higher CSF levels of PAF than patients with minimal or no neurologic involvement (ADC stages 0 or 1) (Gelbard *et al*, 1994). However, high PAF levels in the CSF were also associated with other neurological problems unrelated to AIDS or HIV infection. We found that PAF at doses of approximately 1 ng/ml was quite toxic for the NT2N cells, indicating that it by itself is sufficient for neuronal killing, without a requirement for involvement of

other cell types like astrocytes. PAF is secreted from HIV infected monocytes cocultivated with astroglial cells (Genis *et al*, 1992), and in this system it becomes a prime candidate for HIVD.

Because the neuronal differentiation process is relatively long and dependent on precise cell culture conditions, the cell system described here requires an initial time investment. However, as demonstrated in these experiments, it is a powerful method for demonstrating the toxicity of putative mediators of the neuronal abnormalities characteristic of HIVD. In conjunction with primary microglia and astrocytes, it may provide a substrate for the identification of therapeutic compounds useful in the treatment of this complication of HIV infection.

## Materials and methods

### Cell culture

Human teratocarcinoma cells (NT2 cells) were seeded at  $2 \times 10^6$  cells per T75 flask and were differentiated with 10  $\mu$ M trans-retinoic acid in DMEM 10% FBS with 2 mM glutamine and penicillin-streptomycin for 4 weeks with twice weekly media changes. The cells were then trypsinized and replated overnight in two T-225 flasks with retinoic acid-free media. The following day the cells were trypsinized, replated in T75 flasks and fed with a 1:1 mixture of conditioned media and DMEM 10% FBS with 2 mM glutamine and P/S supplemented with 10  $\mu$ M uridine, 10  $\mu$ M fluorodeoxyuridine, 1  $\mu$ M cytosine arabinoside as mitotic inhibitors. One week later, the cells were again trypsinized and transferred to poly-L-lysine and Matrigel (Becton Dickinson #40234) coated plates with 6, 12, 24, 48 or 96 wells at cell concentrations of  $2 \times 10^6$ ,  $1 \times 10^6$ ,  $5 \times 10^5$ ,  $2 \times 10^5$  or  $1 \times 10^5$  cells per well, respectively. One day prior to seeding the cells, the wells were coated with 10  $\mu$ g/ml sterile poly-L-lysine and left in the hood overnight to dry. The day the cells were plated, the wells were layered with a 1/35 dilution of Matrigel in DMEM media for at least 2–3 h at 37°C. The media was removed immediately prior to plating the cells and replaced with media with mitotic inhibitors as described above. For these experiments, the cells were used between 4–5 weeks after terminal differentiation, a time when there is maximum expression of NMDA receptors.

### Treatment of cells with rgp120, IL-1 $\beta$ , IFN $\gamma$ , TNF $\alpha$ and PAF

The cell culture medium was removed and the cells were rinsed gently with control salt solution (120 mM NaCl/5.4 mM KCl/1.8 mM CaCl $_2$ /25 mM Tris HCl/15 mM glucose, pH 7.4). Cells were then treated with 20 pM to 200 pM rgp120<sub>SF2</sub> or rgp120<sub>BH10</sub>, 100  $\mu$ M IL-1 $\beta$ , 100  $\mu$ M IFN $\gamma$ , 50 ng/ml to 500 ng/ml TNF $\alpha$ , or 100 to 10 000 pg/ml PAF in



serum-free DMEM, 2 mM glutamine and penicillin-streptomycin. Sources of these were: rgp120<sub>BH10</sub>, a gift from R Sweet, SmithKline Beecham; rgp120<sub>SF2</sub>, AIDS Repository, IL1 $\beta$ , (80-3542-01, Genzyme, Boston, MA), IFN $\gamma$ , (a gift from J Fantini), TNF $\alpha$  (Sigma T-0157), PAF, (L-100 Biomol, Plymouth Meeting, PA). Samples of 100  $\mu$ l of supernatant were drawn off cells at 3, 8, 12, 24, 30, 48 and 72 h to be evaluated for LDH activity. All of the experiments were performed at least in triplicate.

#### LDH activity cell toxicity assay

Quadruplicate 5  $\mu$ l samples of each condition were placed in ELISA wells, and the assay for LDH activity was performed as indicated in the manufacturer's instructions (Sigma), with some exceptions. Briefly, pyruvate solution (Sigma # 500L-1) was added to NADH substrate (Sigma #340-105) at 1 mg/ml and mixed. Twenty-five  $\mu$ l were added to each well using a multi-channel pipetter. The plate was covered with parafilm and incubated for 30 min at 37°C. After incubation, 25  $\mu$ l color reagent (Sigma #505-2) was added to each sample and the plate incubated at room temperature for 20 min. Two-hundred  $\mu$ l of 0.4 N NaOH was then added to each sample to stop the reaction, and the plate was read at 450 nm between 5–30 min later. A calibration curve was prepared during each assay, and the LDH activity calculated in units/ml.

#### MTT conversion cell viability assay

Approximately  $1 \times 10^5$  NT2N cells were seeded in each well of a 48-well plate and were treated with TNF $\alpha$  (500 ng/ml) for 24 to 72 h in 200  $\mu$ l DMEM 10%FBS 2 mM glutamine. At the desired time point, 100  $\mu$ l were removed. Twenty  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma #M-2128) 5 mg/ml in PBS sterile filtered were added to each well. The cells were incubated at 37°C for 3–4 h then lysed with 100  $\mu$ l of 10% SDS and 0.1 N HCl overnight at 37°C. Two hundred  $\mu$ l of the lysate were pipetted into a 96 well plate and read at 570 nm to determine relative amount of MTT converted to formazan in the viable cells.

#### Western blot

NT2N cells were harvested, washed, pelleted and lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5) with 1:100 PMSF and 5 mM EDTA for 15 min while rocking at 4°C. RIPA buffer was added at 20  $\mu$ l/10<sup>6</sup> cells. The lysate was spun at 13 000 rpm, the supernatant transferred to a fresh microfuge tube, and the protein concentration measured using the Bio-Rad assay. Fifty ng of each sample in 2 $\times$  Laemmli's buffer were loaded onto SDS-PAGE, resolved overnight and transferred using a semi-dry blotting apparatus to a supported nitrocellulose membrane. Western analysis was performed with mouse anti-human TNFR1 antibody [Austral Biologicals #MR-091-4(55)] at 2  $\mu$ g/ml, mouse anti-human HRP, and detected with ECL detection substrates as per manufacturer's instructions (Amersham). An autoradiogram of the membrane was analyzed for expression of TNFR1 receptor.

#### DNA fragmentation ladder

Cells were lysed with 1 ml TE pH 7.6 and 0.5% SDS then centrifuged for 30 min to pellet the chromatin DNA while leaving the fragmented DNA in the supernatant. Sample DNA was extracted with phenol:chloroform. The sample was then resuspended in 19  $\mu$ l TE, treated with RNase A, run on a 2% agarose gel and visualized with ethidium bromide. Positive controls were provided by DNase treated cells, and by the use of an unrelated virus (La Crosse virus) that causes apoptosis in many cell types (Pekosz *et al*, submitted).

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