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Neuronal apoptosis induced by HIV-1 Tat protein and TNF-α: potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia

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Apoptosis of neurons and non-neuronal cells has been demonstrated in the brain of AIDS patients with dementia. Previous studies suggest that the apoptotic stimuli are likely to be soluble factors. Several candidates for the soluble factors that lead to neuronal apoptosis in HIV-1 infection have been proposed, including the HIV-1 Tat protein and TNF-α. The mechanisms that lead to neuronal apoptosis in the brain of AIDS patients *in vivo*, may involve the combined effects of more than one pro-apoptotic factor. In this study, we examine whether exposure of primary human neurons to the combination of HIV-1 Tat and TNF-α can potentiate the induction of neuronal apoptosis compared with exposure to either factor alone. TNF-α was shown to potentiate the induction of neuronal apoptosis by HIV-1 Tat via a mechanism that involves increased oxidative stress. Antioxidants inhibited, but did not completely abolish the induction of neuronal apoptosis by Tat, suggesting that other mechanisms are also likely to be involved. These findings suggest that soluble HIV-1 Tat and TNF-α may play a role in neuronal apoptosis induced by HIV-1 infection of the CNS, particularly when present in combination. Our findings further suggest that one mechanism whereby combinations of pro-apoptotic factors may potentiate the induction of neuronal apoptosis in the brain of AIDS patients is by increasing oxidative stress. Understanding the role of oxidative stress and other mechanisms that lead to apoptosis in HIV-1 infection of the CNS may advance the development of new therapeutic strategies to prevent neuronal cell death and improve neurologic function in AIDS patients.

Keywords: apoptosis; brain; central nervous system; HIV-1; Tat; TNF-α

HIV-1 infects the central nervous system (CNS) and frequently causes dementia and other neurologic disorders AIDS patients (reviewed in Epstein and Gendelman, 1993; Price, 1996). Most of the HIV-1-infected cells in the brain are macrophages and microglia (Gabuzda *et al*, 1986; Kure *et al*, 1991; Epstein and Gendelman *et al*, 1993; Brew *et al*, 1995; Takahashi *et al*, 1996). Astrocytes and capillary endothelial cells may also be infected at a very low level (Bagasra *et al*, 1996; Takahashi *et al*, 1996). The neuropathological abnormalities associated with HIV-1 encephalitis (HIVE) include brain atrophy, reactive gliosis, demyelination, microglial nodules, multinucleated giant cells, evidence of abnormal blood-brain barrier permeability and neuronal loss. (Navia *et al*, 1986; Ketzler *et al*, 1990; Kure *et al*, 1991; Wiley *et al*, 1991; Masliah *et al*, 1992; Glass *et al*, 1993; Brew *et al*, 1955).

Apoptosis of neurons and non-neuronal cells (i.e. astrocytes, endothelial cells, and macrophages/microglia) has been demonstrated in autopsy brain tissue from pediatric and adult AIDS patients with clinical encephalopathy or dementia. (Table 1). Apoptosis has also been demonstrated in the brain of macaques infected with simian immunodeficiency virus (Adamson *et al*, 1996) and SCID mice engrafted with HIV-1-infected monocytes (Persidsky *et al*, 1996). Neuronal apoptosis occurs during normal CNS development, but in the adult brain is only associated with certain pathological condi-

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tions, (e.g., Alzheimer's disease, amyotrophic lateral sclerosis, stroke). Together, these observations suggest that apoptosis of neurons, and possibly other cell types, is a likely cause of CNS injury leading to cognitive dysfunction and other neurologic abnormalities in AIDS patients.

The mechanisms that lead to neuronal apoptosis in the brain of AIDS patients are poorly understood. Several lines of evidence suggest that neuronal apoptosis is induced by soluble factors acting at a distance rather than by direct viral infection. Neurons are not directly infected by HIV-1. Furthermore, the majority of apoptotic neurons are not localized adjacent to the HIV-1-infected cells (Adie-Biassette *et al*, 1995; Shi *et al*, 1996; Vallat *et al*, 1998). Moreover, apoptosis in HIV-1-infected primary human brain cultures *in vitro* is not significantly induced until 1 to 2 weeks after the time of peak viral infection (Shi *et al*, 1996). Several candidates for soluble pro-apoptotic factors that may lead to neuronal cell death in HIV-1 infection have been proposed based on *in vitro* studies (Table 1). These include soluble forms of the HIV-1 gp120 and Tat proteins, and factors secreted by infected or activated macrophages and microglia, such as TNF-α, oxygen free radicals, nitric oxide, excitatory amino acids, and other yet unknown factors (Sabatier *et al*, 1991; Genis *et al*, 1992; MüIler *et al*, 1992; Epstein and Gendelman, 1993; Gelbard *et al*, 1993; Lipton and Gendelman, 1995; Magnuson *et al*, 1995; Talley *et al*, 1995; Gulian *et al*, 1996; New *et al*, 1997). However, the *in vivo* role of these factors in contributing to neuronal apoptosis in the brain of AIDS patients has not been established.

The mechanisms that lead to neuronal apoptosis in the brain of AIDS patients *in vivo* may involve combined effects of more than one pro-apoptotic factor. We hypothesized that exposure of neurons to the combination of HIV-1 Tat protein and TNF-α would potentiate the induction of neuronal apoptosis compared to exposure to either factor alone. Previous studies have demonstrated that soluble Tat and TNF-α are neurotoxic (Sabatier *et al*, 1991; Gelbard *et al*, 1993; Magnuson *et al*, 1995; Nath *et al*, 1996) and can induce apoptosis in primary human neurons or neuroblastoma cell lines (Talley *et al*, 1995; New *et al*, 1997), as well as other cell types *in vitro* (Li *et al*, 1995; Purvis *et al*, 1995; Westendorp *et al*, 1995a,b; Ehret *et al*, 1996). HIV-1 Tat activates transcription driven by the HIV-1 LTR, as well as certain cellular genes (reviewed in Chang *et al*, 1995). These effects can be mediated by an extracellular soluble form of Tat, which can be released from HIV-1 infected cells and taken up by neuronal and non-neuronal cells (Sabatier *et al*, 1991; Chang *et al*, 1995; Magnuson *et al*, 1995; Ma and Nath, 1997). Tat peptides which contain the basic region with the first exon amino acid sequence are also neurotoxic (Sabatier *et al*, 1991; Hayman *et al*, 1993; Weeks *et al*, 1995; Nath *et al*, 1996; Philippon *et al*, 1994). TNF-α is produced by HIV-1-infected macrophages and microglia, particularly when co-cultured with astrocytes (Genis *et al*, 1992). Increased levels of TNF-α have been shown to correlate with clinical dementia in AIDS patients (Tyor *et al*, 1992; Glass *et al*, 1993; Wesselingh *et al*, 1993; Nuovo and Alfieri, 1996). TNF-α can potentiate the induction of lymphocyte apoptosis by Tat (Westendorp *et al*, 1995b), but their combined effects on neural cells have not been examined.

To determine whether TNF-α can potentiate the induction of neuronal apoptosis by soluble HIV-1 Tat, we examined neuronal apoptosis in primary human fetal brain cultures induced by recombinant Tat protein (full-length HIV-1 Tat amino acids 1–86, Immunodiagnostics, Inc., lot #T-1) in the presence or absence of TNF-α. The preparation of the primary brain cultures, which contain a mixture of neurons (10–30%), astrocytes (70–90%), microglia (1–5%), and fibroblasts (1–5%), from 13–18 weeks gestation fetal abortuses has been described (Busciglio *et al*, 1993). Tissue was procured using an approved protocol in compliance with institutional and federal regulations. The cultures (100 000

Table 1 Summary of *in vitro* and *in vivo* studies on apoptosis in HIV-1 infection of the CNS

In vivo *studies*	
Petito and Roberts, 1995	Apoptotic neurons, astocytes, and multinucleated giant cells in the brain of adults with HIVE
Gelbard *et al*, 1995	Apoptotic neurons, macrophages, and microglia in the brain of children with HIVE
Adie-Biassette *et al*, 1995	Apoptotic neurons, and perivascular cells in the brain of adults with HIVE
Shi *et al*, 1996	Apoptotic neurons, astrocytes, and endothelial cells in the brain of adults with HIVE
An *et al*, 1996	Apoptotic neurons and glial cells in the brain of HIV-1 positive AIDS and pre-AIDS patients
Krajewski *et al*, 1997	Altered expression of Bcl-2, Bcl-x, and Bax in the brain of children with HIVE
Vallat *et al*, 1998	Apoptotic neurons, astrocytes, endothelial cells, pericytes, and macrophages in the brain of children with AIDS
In vitro *studies*	
Shi *et al*, 1996	Apoptosis of neurons and astrocytes in primary human brain cultures infected with HIV-$1_{89.6}$
New *et al*, 1997	Apoptosis of primary human neurons induced by soluble HIV-1 Tat protein
Talley *et al*, 1995	Apoptosis of differentiated SK-N-MC human neuroblastoma cells induced by TNF-α
Müller *et al*, 1992	Apoptosis of neurons in rat cortical cultures induced by soluble HIV-1 gp120 protein

to 200 000 cells per well in 24-well plates) were maintained in DMEM containing 10% calf serum for 10–12 days prior to treatment with soluble Tat for 72 h followed by fixation in 4% paraformaidehyde. Apoptotic cells were detected *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Apoptag kit, Oncor) in combination with immunofluorescence staining with the neuron-specific marker mouse monoclonal anti-microtubule-associated protein-2 (MAP-2, 1 : 50 dilution, Sigma) or rabbit anti-Tau (1 : 100 dilution, Dako) as described (Shi *et al*, 1996). In a previous study, we demonstrated that the detection of TUNEL-positive cells by this method correlates with the detection of apoptotic nuclear morphology by electron microscopy or staining with propidium iodide (Shi *et al*, 1996).

In initial experiments, we performed TUNEL staining in combination with Tau or MAP-2 immunofluorescence staining after 24, 48 and 72 h of treatment with recombinant Tat and found that induction of neuronal apoptosis was maximal at 72 h of exposure (Figure 1 and not shown). Exposure to soluble Tat at 1 and 10 μg/ml induced a dose-dependent loss of neurons to 83% and 76% of the level in untreated control cultures, respectively (Figure 2, left). Quantitation of the TUNEL-positive nuclei demonstrated that soluble Tat at 1 and 10 μg/ml induced apoptosis to 169% and 291% of the level in untreated control cultures, respectively (Figure 2, right). The percentage of TUNEL-positive cells in untreated control cultures ranged from 0.5–1.5% *versus* 2.5 10% in cultures treated with Tat (1 to 10 μg/ml) among independent experiments using cultures from different tissue donors. Recombinant human TNF-α (1 ng/ml, Boehringer Mannheim) potentiated the induction of neuronal loss and apoptosis by Tat (Figure 2). The combination of Tat (1 μg/ml) and TNF-α (1 ng/ml) induced apoptosis detected by TUNEL staining to 491% of the level in untreated control cultures, whereas Tat or TNF-α alone at the same concentrations induced apoptosis to 169% or 138% of control levels, respectively (Figure 2, right). Similar results were obtained when propidium iodide staining was performed to demonstrate condensed or fragmented apoptotic nuclear morphology as described (Shi *et al*, 1996) (Figures 3 and 4), providing further evidence that TNF-α potentiates the induction of neuronal apoptosis by Tat.

Oxidative stress induces apoptosis in neurons (Kane *et al*, 1993; Greenlund *et al*, 1995; Busciglio and Yankner, 1995; Bonfoco *et al*, 1995), as well as other cell types. Previous studies in non-neuronal cells have shown that exposure to either HIV-1 Tat or TNF-α increases cellular levels of oxygen free radicals (Westendorp *et al*, 1995b; Ehret *et al*, 1996). These observations raise the possibility that the

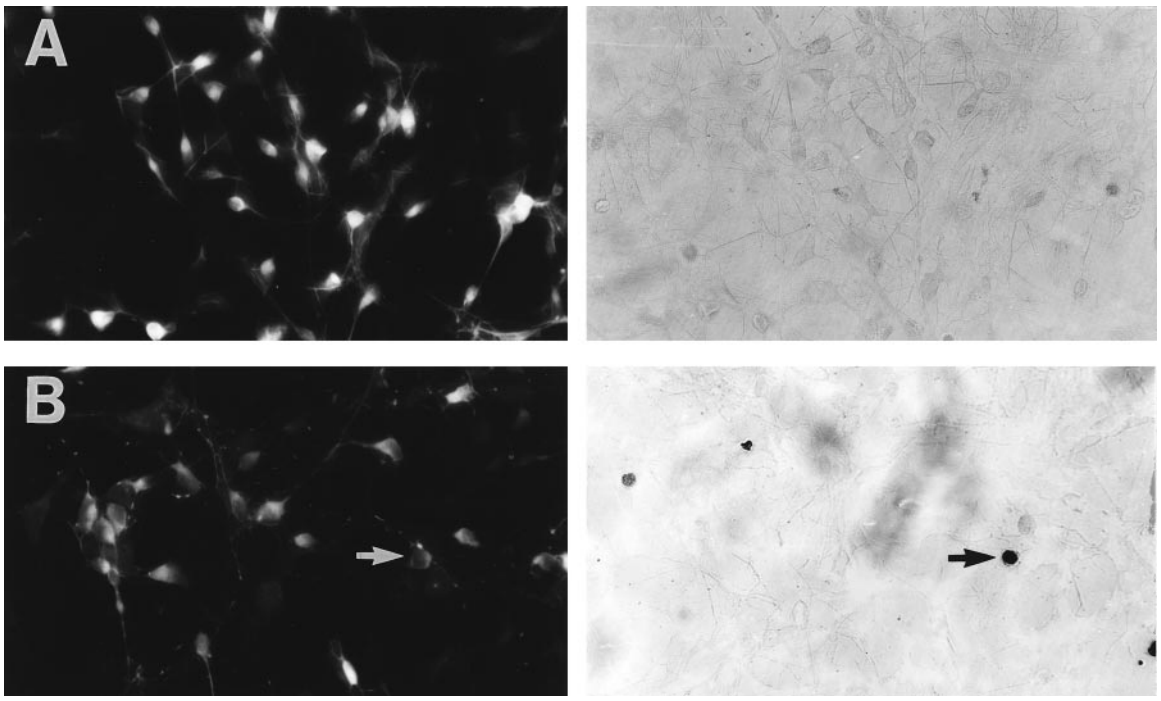

Figure 1 Apoptosis of neurons in primary human brain cultures exposed to recombinant HIV-1 Tat protein (10 μg/ml) for 72 h. Combined TUNEL (right panels) and anti-Tau immunofluorescence (left panels) staining of untreated control (**A**) and Tat-treated (**B**) cultures demonstrates apoptosis in neurons (arrow), loss of Tau staining, and degeneration of neuritic processes in Tat-treated cultures. Results are representative of three independent experiments.

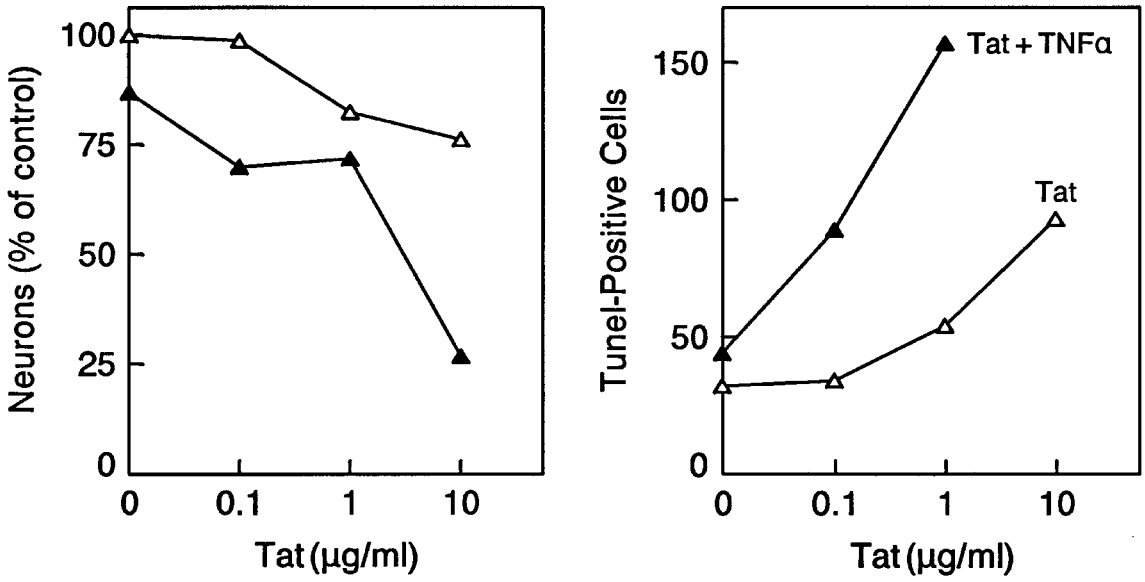

Figure 2 TNF-α potentiates neuronal apoptosis induced by exposure of primary human brain cultures to HIV-1 Tat. Quantitation of neuronal loss (left) and TUNEL-positive cells (right) in primary human brain cultures exposed to different concentrations of recombinant HIV-1 Tat protein for 72 h in the presence (dark triangles) or absence (white triangles) of TNF-α (1 ng/ml). TNF-α was added 24 h after addition of Tat. Left panel: the number of neurons was quantitated by counting Tau-positive cells in 10 random fields using a 20× objective. The labeling symbols are identical to those shown in the right panel. For the cultures treated with Tat and TNF-α, the y axis is crossed at 87%, since TNF-α alone at 1 ng/ml induced a minor decrease in neuronal viability compared to untreated control cultures. Right panel: the number of TUNEL-positive cells was quantitated by counting TUNEL-positive cells in 20 random fields using a 20× objective. Combined TUNEL and anti-Tau immunofluorescence staining confirmed TUNEL-positive cells that were double stained with anti-Tau (see Figure 1). Results are representative of three independent experiments.

combined stimulatory effects of Tat and TNF-α on oxygen free radical production may be a mechanism whereby TNF-α potentiates the induction of neuronal apoptosis by Tat. To determine the effect of Tat and TNF-α on the generation of oxygen free radicals in neural cells, we exposed live primary human brain cultures to the redox-sensitive fluorescent dye 2,7 dichlorofluorescein diacetate (DCFDA) (10 μM) for 1 h at 37°C as described (Busciglio and Yankner, 1995) following treatment with Tat or TNF-a alone or in combination. DCFDA is cell permeable and interacts with reactive oxygen species to generate a fluorescent product, 2,7 dichlorofluorescein (DCF), that can be visualized *in situ* by fluorescence microscopy with a fluorescein wavelength filter, or quantitated by flow cytometry (Kane *et al*, 1993; Busciglio and Yankner, 1995). Cultures treated with Tat (10 μg/ml), or with the combination of Tat (1 μg/ml) and TNF-α (1 ng/ml) showed an increase in the number of DCF-positive cells visualized by fluorescence microscopy compared to untreated control cultures (Figure 5). Quantitation of the mean fluorescence intensity by flow cytometry as described (Busciglio and Yankner, 1995) demonstrated that cultures treated with Tat alone (1 μg/ml) or TNF-α alone (1 ng/ml) showed an 8% and 34% increase in DCF fluorescence, respectively, while cultures treated with Tat (1 μg/ml) and TNF-α (1 ng/ml) in combination showed a 64% increase in DCF fluorescence. Exposure of cultures to Tat alone at a higher concentration (10 μg/ml) showed a 19% increase in DCF fluorescence. Non-viable cells were excluded by staining of the live cultures with prodium iodide and gating out the propidium iodide-positive cells (Busciglio and Yankner, 1995). These results suggest that exposure of primary brain cultures to HIV-1 Tat or TNF-α induces the generation of reactive oxygen species. Furthermore, this pro-oxidant effect is potentiated when the cultures are exposed to Tat and TNF-α in combination.

To test whether antioxidants can inhibit neuronal apoptosis induced by soluble HIV-1 Tat, primary brain cultures were preincubated with the antioxidant drugs phenylbutylnitrosulfone (PBN) (100 μM), N-acetylcysteine (NAC) (100 μM), or the combination of catalase and superoxide dismutase (CAT/SOD) (20 μg/ml of each) (Sigma) for 1 h prior to treatment with Tat (10 μg/ml) for 72 h. PBN is a free radical spin trap, NAC is both a glutathione precursor and free radical scavenger, and CAT/SOD degrade hydrogen peroxide and superoxide anions. Treatment with PBN, NAC or CAT/SOD inhibited the induction of apoptosis by Tat to 26, 35, and 42%

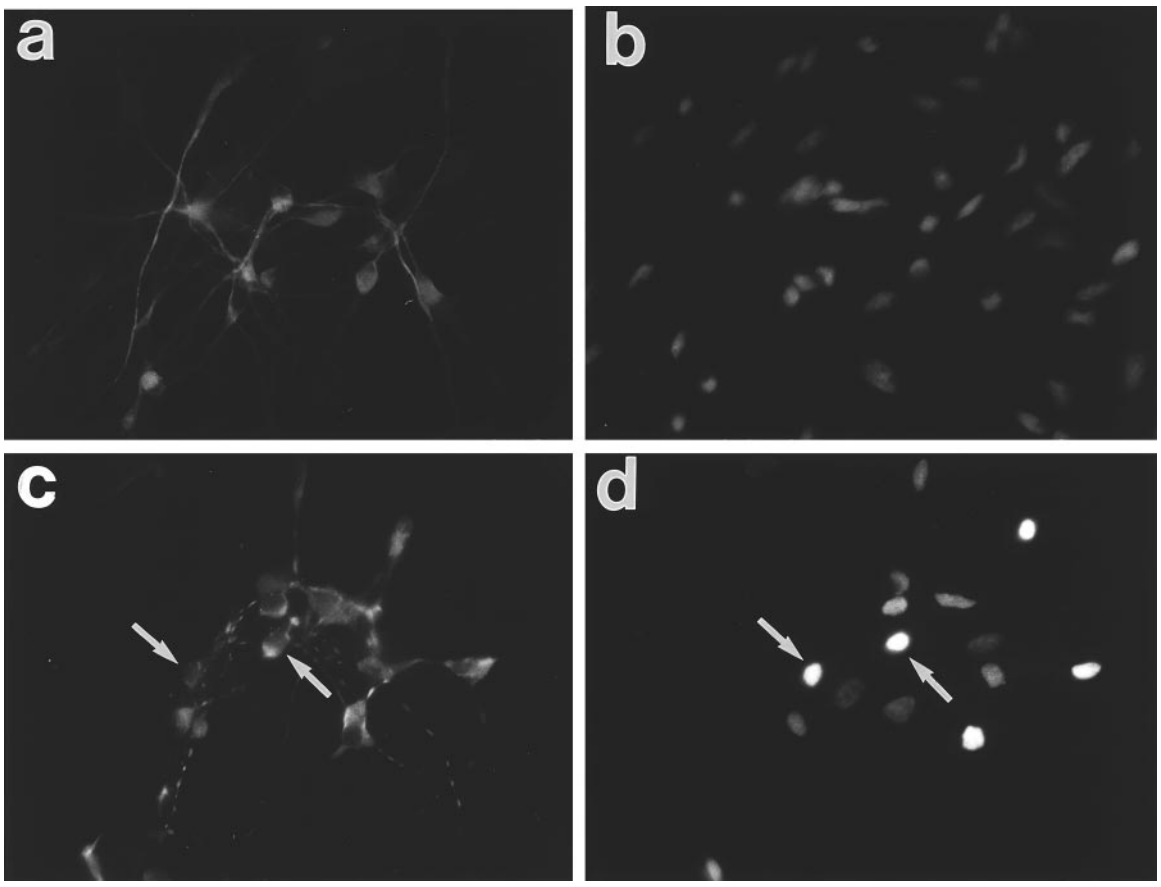

Figure 3 Detection of apoptotic nuclear morphology in primary human neurons exposed to HIV-1 Tat and TNF-α. Primary human brain cultures were exposed to recombinant HIV-1 Tat protein (1 μg/ml) and TNF-α (1 ng/ml) in combination for 72 h as described in Figure 2. Combined propidium iodide (right panels) and anti-MAP-2 immunofluorescence staining detected with a fluorescein-conjugated secondary antibody (left panels) of untreated control cultures (**a** and **b**) and cultures exposed to Tat and TNF-α in combination (**c** and **d**) visualized with rhodamine- and fluorescein-specific filters, respectively. Apoptotic condensed nuclear morphology (arrows) and degeneration of neuritic processes is shown in cultures exposed to Tat and TNF-α in combination (**c** and **d**).

of the level induced in untreated control cultures, respectively, as determined by propidium iodide staining and counting the percentage of nuclei with apoptotic morphology (Figure 6). These results provide further evidence that oxidative stress contributes to the induction of neuronal apoptosis by HIV-1 Tat.

Our studies provide evidence that soluble forms of HIV-1 Tat and TNF-α may play a role in neuronal apoptosis induced by HIV-1 infection of the CNS, particularly when present in combination. TNF-α was shown to potentiate the induction of neuronal apoptosis by HIV-1 Tat via a mechanism that involves increased production of oxygen free radicals. These results suggest that one mechanism whereby combinations of pro-apoptotic factors may potentiate the induction of neuronal apoptosis in the brain of AIDS patients is by increasing oxidative stress. HIV-1 Tat induces cellular transcription of TNF-α (Chang *et al*, 1995; Chen *et al*, 1997), while TNF-α can upregulate expression of HIV-1 Tat by increasing expression of NF-kappa B (Westendorp *et al*, 1995b). Thus, increased expression of Tat or TNF-α can lead to a cycle resulting in increased steady state levels of both Tat and TNF-α, which may further increase oxidative stress. In view of this, we cannot exclude the possibility that the neurotoxicity observed in cultures treated with Tat may in part be due to upregulation of TNF-α (Chen *et al*, 1997). Our finding that soluble HIV-1 Tat is neurotoxic and induces apoptosis in primary human neurons is consistent with previous studies (Magnuson *et al*, 1995; New *et al*, 1997). The finding that TNF-α alone at 1 ng/ml causes minimal neurotoxicity to primary human neurons contrasts with a study by Gelbard *et al*, (1993) which found TNF-α neurotoxicity at $\geqslant$200 pg/ml. The different results obtained by Gelbard *et al* (1993) may reflect differences in the primary brain culture conditions, such as the length of time after plating (10–12 *versus* 28 days), different cell culture media (10% calf serum *versus* serum-free medium supplemen-

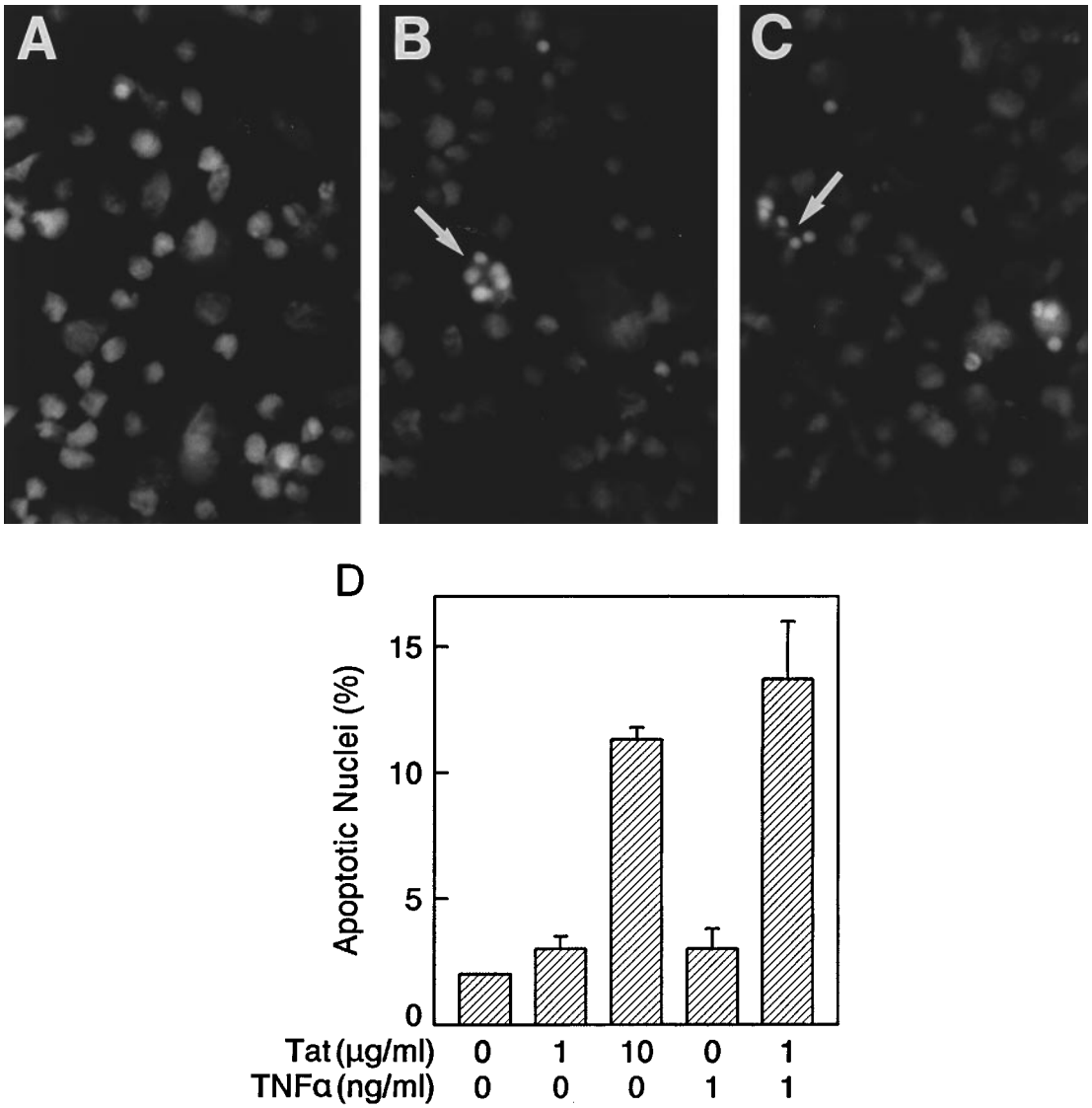

Figure 4 Apoptosis detected by propidium iodide staining of nuclei in primary human brain cultures exposed to HIV-1 Tat and TNF-α. (**A**) Untreated control cultures. (**B** and **C**) Cultures exposed to recombinant HIV-1 Tat protein (10 μg/ml) (**B**) or the combination of Tat (1 μg/ml) and TNF-α (1 ng/ml) (**C**) for 72 h. Apoptotic nuclear morphology was detected by propidium iodide staining as described (Shi *et al*, 1996). (**D**) Apoptosis was quantitated by counting the percentage of cells with apoptotic nuclear morphology (arrows) in at least 250 nuclei in 10 random fields using a 40× objective (mean ± s.d., n=2). Results are representative of two independent experiments.

ted with N1 components), or the relative percentage of astrocytes (70–90% *versus* <30%). Most likely, these or other methodological variables influenced the vulnerability of primary brain cultures to TNF-α neurotoxicity. In particular, the high percentage of astrocytes in our cell culture model is likely to have a neuroprotective effect, since glutamate uptake is a major function of astrocytes. A critical question which remains to be determined is whether the levels of Tat or TNF-α present in brain or cerebrospinal fluid *in vivo* could reach the levels required to induce apoptosis. The levels of Tat present in the serum of AIDS patients are 10–100 ng/ml (Westendorp *et al*, 1995a). The higher concentrations of Tat required to induce apoptosis in our studies and in studies by other groups (Sabatier *et al*, 1991; Magnason *et al*, 1995; Westendorp *et al*, 1995b) may reflect the reduction in biological activity which can occur when recombinant Tat protein becomes oxidized (McCloskey *et al*, 1997 and references therein).

Our studies suggest that TNF-α is one of several factors that can act to potentiate HIV-1-related neuronal injury. The demonstration that increased levels of TNF-α in the brain of AIDS patients correlates with clinical dementia is

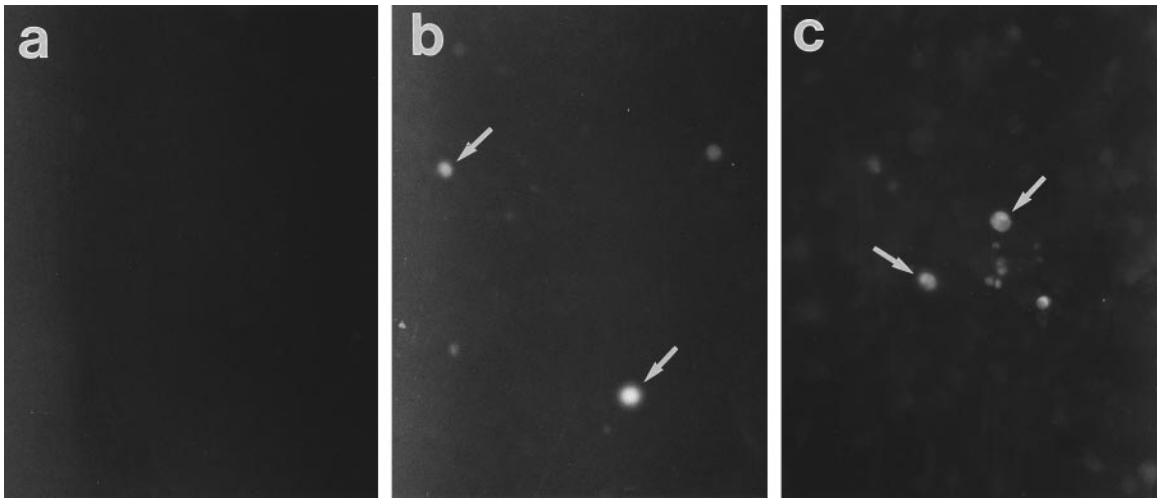

Figure 5 Increased production of oxygen free radicals demonstrated by DCF fluorescence in primary human brain cultures exposed to HIV-1 Tat and TNF-α. (**a**–**c**) DCF-positive cells (arrows) visualized by fluorescence microscopy in untreated control cultures (**a**) and in cultures treated with recombinant HIV-1 Tat protein (10 μg/ml) (**b**) or the combination of Tat (1 μg/ml) and TNF-α (1 ng/ml) (**c**) for 72 h.

consistent with this possibility (Glass *et al*, 1993; Wesselingh *et al*, 1993; Nuovo and Alfieri, 1996). Our finding that both HIV-1 Tat and TNF-α can increase oxidative stress in primary brain cultures is consistent with previous studies in neurally-derived cell lines and non-neuronal cells (Talley *et al*, 1995; Westendorp *et al*, 1995b; Ehret *et al*, 1996). A previous study suggested that Tat and TNF-α can increase oxidative stress by downregulation of manganese-dependent SOD in T cells (Westendorp *et al*, 1995b), but whether this mechanism occurs in neuronal cells remains to be determined. We found that antioxidants inhibited, but did not completely abolish the induction of neuronal apoptosis by Tat. Thus, other mechanisms such as neurotoxicity mediated through excitatory amino acid receptors are also likely to be involved (Magnuson *et al*, 1995; Nath *et al*, 1996). Excitatory amino acid receptors have also been implicated in mediating neurotoxic effects of TNF-α (Gelbard *et al*, 1993). Furthermore, TNF-α has been shown to inhibit glutamate uptake by astrocytes (Fine *et al*, 1996). The over-activation of NMDA receptors by glutamate or other excitatory amino acids can cause oxidative stress, while oxygen free radicals can cause secondary glutamate neurotoxicity by increasing glutamate release and inhibiting glutamate uptake (Coyle and Puttfarcken, 1993; Dugan and Choi, 1994; Beal, 1995). Either oxidative stress or chronic NMDA receptor activation can lead to neuronal apoptosis (Kane *et al*, 1993; Greeunlund *et al*, 1995; Bonfoco *et al*, 1995). *In vitro* studies suggest that mild insults cause apoptotic neuronal cell death, whereas intense insults cause necrotic neuronal cell death (Bonfoco *et al*, 1995). Together, these observations suggest that NMDA receptor-mediated neurotoxicity and oxidative stress can interact in a sequential or reinforcing manner leading to apoptotic neuronal cell death.

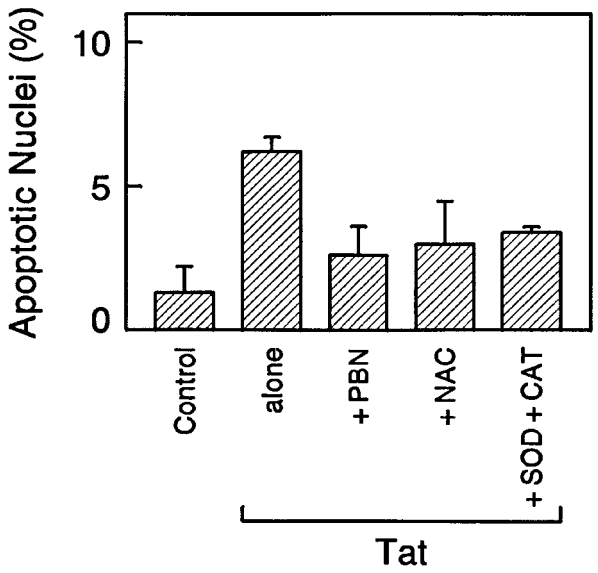

Figure 6 Antioxidants inhibit apoptosis in primary human brain cultures exposed to HIV-1 Tat. Cultures were preincubated with PBN (100 μM), NAC (100 μM), or CAT/SOD (20 μg/ml each) for 24 h prior to addition of recombinant HIV-1 Tat protein (10 μg/ml) for 72 h. Fresh drug was added after 48 h. Apoptotic nuclear morphology was detected by staining with propidium iodide and quantitated by counting the percentage of apoptotic nuclei as in Figure 4 (mean ± s.d., n=2). $P<0.05$ by Student's t test for cultures treated with PBN, NAC, or CAT/SOD compared with untreated control cultures.

The identification of soluble factors and other mechanisms that lead to neuronal apoptosis in the brain of AIDS patients *in vivo* is an important area for future investigation. In this regard, it will be

important to elucidate the *in vivo* role of soluble factors such as the HIV-1 Tat and gp120 proteins, TNF-α, and other yet unknown factors (Gulian *et al*, 1996) released by activated or HIV-1-infected macrophages and microglia as initial triggers for neuronal apoptosis, and to identify the neuronal receptor(s) that initiate the apoptotic pathway. It will also be important to determine the role of oxidative stress and excitatory amino acids as final common pathways leading to neuronal apoptosis in AIDS patients, and whether antioxidants or NMDA receptor antagonists can prevent neuronal cell death *in vivo*. Clinical studies have suggested that glutathione levels are depleted in the blood and cerebrospinal fluid of AIDS patients, providing indirect evidence for increased oxidative stress in the CNS (Castagna *et al*, 1995). Understanding mechanisms that lead to neuronal apoptosis in HIV-1 infection of the CNS may advance the development of new therapeutic strategies to prevent neuronal cell death and improve neurologic function in AIDS patients.

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