Published in final edited form as:

Neurobiol Dis. 2007 June ; 26(3): 661–670.

Human Immunodeficiency Virus Type-1 Protein Tat Induces Tumor Necrosis Factor- α -Mediated Neurotoxicity

Lara Buscemi, David Ramonet, and Jonathan D. Geiger^{*}

Department of Pharmacology, Physiology and Therapeutics, University of North Dakota, School of Medicine and Health Sciences, Grand Forks, North Dakota, 58202

Abstract

HIV-1 infection causes, with increasing prevalence, neurological disorders characterized in part by neuronal cell death. The HIV-1 protein Tat has been shown to be directly and indirectly neurotoxic. Here, we tested the hypothesis that a non-neurotoxic epitope of Tat can, through actions on immune cells, increase neuronal cell death. Tat₁₋₇₂ and a mutant Tat₁₋₇₂ lacking the neurotoxic epitope (Tat Δ ₃₁₋₆₁) concentration-dependently and markedly increased TNF- α production in macrophage-like differentiated human U937 and THP-1 cells, in mouse peritoneal macrophages and in mouse brain microglia. Tat₁₋₇₂ but Tat Δ ₃₁₋₆₁ was not neurotoxic when applied directly to neurons. Supernatants from U937 cells treated with either Tat₁₋₇₂ or Tat Δ ₃₁₋₆₁ were neurotoxic and their immunoneutralization with an anti-TNF- α antibody decreased Tat₁₋₇₂- and Tat Δ ₃₁₋₆₁-induced neurotoxicity. Together, these results demonstrate that the neurotoxic epitope of Tat₁₋₇₂ is different from the epitope that is indirectly neurotoxic following production of TNF- α from immune cells, and suggest that therapeutic interventions against TNF- α might be beneficial against HIV-1 associated neurological disorders.

Keywords

TNF- α ; HIV-1; Tat; neurotoxicity; macrophages; microglia; U937 cells; THP-1 cells; inflammation

Introduction

HIV-1 associated dementia, a clinical condition marked by ataxia, memory loss, and motor and behavioral abnormalities is increasing in prevalence among HIV-1 infected people and remains an important manifestation of HIV-1 infection (Bouwman et al., 1998; Sacktor et al., 2002; McArthur, 2004; Nath and Berger, 2004; Kaul et al., 2005). Pathological features of HIV-1 associated dementia include neuron dysfunction and death, demyelination, astrogliosis, and inflammation (Wiley et al., 1998; Rappaport et al., 1999). The pathogenesis of HIV-1 associated dementia however is complex, ill-understood and multiple mechanisms have been implicated in part because neurons are not infected by HIV-1 (Takahashi et al., 1996) and viral burden does not correlate with disease onset or progression (Glass et al., 1995; Ellis et al., 1997; Wiley et al., 1998).

^{*}To whom correspondence should be addressed: Jonathan D. Geiger, Ph.D., Department of Pharmacology, Physiology and Therapeutics, University of North Dakota School of Medicine and Health Sciences, 501 N. Columbia Road, Grand Forks, North Dakota 58203, (701) 777-2183 (P), (701) 777-4490 (F), jgeiger@medicine.nodak.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Closely linked with the development and progression of HIV-1 associated dementia are early-on infiltration of infected T cells and monocytes, and later recruitment of uninfected macrophages into the CNS. One of the most important clinical and pathological correlates of HIV-associated dementia is the infiltration of activated macrophages into the CNS and the activation of microglia (Brew et al., 1995; Glass et al., 1995; Glass and Wesselingh, 2001). Once recruited, peripheral immune cells as well as activated brain-resident cells produce a variety of inflammatory mediators, including tumor-necrosis factor- α (TNF- α) (Tyor et al., 1992; Glass et al., 1993; Wesselingh et al., 1993; Glass et al., 1995; Wesselingh et al., 1997; Glass and Wesselingh, 2001). In addition to the direct effects of HIV-1 virotoxins on neurons, neurotoxic effects can be mediated through the abnormal production, release or relative amounts of cytokines in brain (Nath and Geiger, 1998). TNF- α has been implicated in the pathogenesis of HIV-associated dementia, because elevated levels of TNF- α are present in the serum, CSF and brains of demented AIDS patients (Grimaldi et al., 1991; Glass et al., 1993; Wesselingh et al., 1993), and the severity of dementia is closely correlated with plasma and brain TNF- α levels (Glass et al., 1993; Wesselingh et al., 1993; Seigny et al., 2004). TNF- α may contribute to HIV-1 associated dementia by inducing neuronal dysfunction and death and by synergizing with viral proteins and excitatory amino acids (Shi et al., 1998; Saha and Pahan, 2003).

Tat is a non-structural HIV-1 protein that is produced and secreted by HIV-infected cells, that controls HIV-1 replication through binding and transactivation of its long terminal repeats (Ensoli et al., 1993), and that binds to and activates the transcription of host genes such as TNF- α (Buonaguro et al., 1992; Sawaya et al., 1998). Evidence linking Tat to HIV-1 associated dementia includes findings that Tat increases monocyte/macrophage infiltration into brain, damage to brain-resident cells, and brain levels of TNF- α (Philippon et al., 1994; Jones et al., 1998; Nath and Geiger, 1998; Rappaport et al., 1999; Pu et al., 2003; Saha and Pahan, 2003). Further linking Tat to the pathogenesis of HIV-1 associated dementia, as we and others have shown, are findings that Tat decreases neuronal cell function and viability by directly activating neurons, by dysregulation of intracellular levels of calcium, by increasing excitotoxicity, and by synergizing with harmful effects of pro-inflammatory cytokines, other HIV-1 proteins, and drugs of abuse (Sabatier et al., 1991; Magnuson et al., 1995; Nath et al., 1996; New et al., 1997; Cheng et al., 1998; Jones et al., 1998; Kruman et al., 1998; New et al., 1998; Shi et al., 1998; Haughey et al., 1999; Bonavia et al., 2001; Gurwell et al., 2001; Maragos et al., 2002; Prendergast et al., 2002; Self et al., 2004b; Perry et al., 2005). In addition to the direct toxic effects of Tat on neurons, Tat can damage neurons indirectly through the release of toxins from macrophages and glial cells. However, to date, little has been done to distinguish direct neurotoxic from indirect immune cell-mediated Tat neurotoxicity.

We and others have demonstrated previously that Tat can increase TNF- α release from macrophages and glial cells (Chen et al., 1997; Nath et al., 1999; Mayne et al., 2000; Bruce-Keller et al., 2001; Bennasser et al., 2002; Fotheringham et al., 2004). Tat is encoded by two exons, is up to 104 amino acids in length, and has sequences classified into 5 distinct domains; N-terminal (Tat₁₋₂₁), cysteine-rich (Tat₂₂₋₃₇), core (Tat₃₈₋₄₈), basic (Tat₄₉₋₅₇), and C-terminal (Tat₅₈₋₁₀₄). Tat derived from the first exon (Tat₁₋₇₂) is sufficient to induce TNF- α production (Chen et al., 1997). The N-terminal region of Tat has been identified as the one that contains the active domain for TNF- α production, since Tat₁₋₇₂, Tat₁₋₅₅ and Tat₁₋₁₀₁ induce similar amounts of TNF- α , but the regions between amino acids 20-72 and 30-72 cannot stimulate TNF- α production by monocytes (Bennasser et al., 2002). Other studies suggest that the basic region and the C-terminal region of Tat seem not to be involved in the production of TNF- α (New et al., 1998). Recently, HIV-1 Tat₁₋₇₂ lacking residues 31-61, where the basic region is located, has been used as a control peptide for the neurotoxic actions of Tat₁₋₇₂ (Gurwell et al., 2001; Prendergast et al., 2002; Aksenov et al., 2003; Toborek et al., 2003; Self et al., 2004a; Flora et al., 2005; Perry et al., 2005). In preliminary studies, we noted that Tat _{Δ 31-61} increased

markedly the production of TNF- α by differentiated U937 cells, providing us with a tool to explore Tat's ability to indirectly affect neuronal viability. Given previous findings that HIV-1 Tat induced increases in TNF- α can cause neuronal cell death (New et al., 1998), we designed experiments to determine the extent to which Tat₁₋₇₂ and mutant Tat _{Δ 31-61} could increase the production of TNF- α from immune cells in order to test the hypothesis that Tat-induced increases in TNF- α levels from inflammatory cells were sufficient to cause neuronal cell death.

Materials and Methods

Materials

RPMI-1640 medium and heat-inactivated fetal bovine serum (FBS) were obtained from Atlanta Biologicals (Lawrenceville, GA). Dulbecco's Modified Eagle's Medium (DMEM), AIM-V™ medium, Neurobasal™ medium, B-27 supplement, trypsin and L-glutamine were obtained from GIBCO-Invitrogen (Grand Island, NY). Antibiotic/antimycotic (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μ g/ml amphotericin B), phorbol-12-myristate-13-acetate (PMA), and protein G agarose beads were obtained from Sigma (St Louis, MO). Glass-bottom dishes were from MatTek (Ashland, MA). Highly purified recombinant Tat₁₋₇₂ and Tat _{Δ 31-61} were prepared as previously described (Ma and Nath, 1997) and kindly provided to us by Dr. A. Nath (Johns Hopkins University, Baltimore, MD). The antibody against Tat was obtained from Dr. J. Karn through the NIBSC Centralized Facility for AIDS Reagents supported by EU Programme EVA (contract QLKZ-CT-1999-00609) and the UK Medical Research Council. Human TNF- α ELISA antibodies, streptavidin-alkaline phosphatase and human recombinant TNF- α were from BD Biosciences-Pharmingen (San Diego, CA). Murine TNF- α was measured with an ELISA kit from eBioscience (San Diego, CA). TNF- α neutralizing antibody was from R&D Systems (Minneapolis, MN). Rat anti-mouse CD11b was from Serotec (Raleigh, NC). Hoechst 33342, calcein-AM, and ethidium homodimer-1 were obtained from Molecular Probes-Invitrogen (Eugene, OR).

Animal care

All procedures were reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee. C57BL/6 mice were obtained from Charles River Labs (Wilmington, MA) and the National Cancer Institute (Frederick, MD) and were housed in our Center for Biomedical Research.

Cell and Tissue Culture

Human pro-monocytic U937 (ATCC # CRL-1593.2) and human monocytic leukemia THP-1 cells (ATCC # TIB-202) were maintained in RPMI-1640 media supplemented with 10% heat-inactivated FBS and antibiotic/antimycotic at 37°C and 5% CO₂. U937 and THP-1 cells were plated at a density of 1 $\times$ 10⁶ cells/ml in AIM-V™ serum-free medium and were allowed to differentiate in the presence of 10 ng/ml PMA for 24 h. The media was replaced with fresh AIM-V™ prior to initiation of experiments.

Microglial cultures were prepared three separate times each time using brains from three newborn C57BL/6 mice. After decapitation, brains were removed, meninges were removed and cerebral cortices were mechanically disrupted, trypsinized, and cells were plated in DMEM supplemented with 10% heat-inactivated FBS and antibiotic/antimycotic and incubated for 7-10 days at 37°C and 5% CO₂ until mixed glial cultures were confluent. To isolate the microglia, the confluent mixed glial cultures were subjected to mild trypsinization to remove the astrocytes as described (Saura et al., 2003). The remaining attached microglial cells were tested for purity with CD11b immunostaining and re-plated using the DMEM media one day before experiments were performed at a density of 3 $\times$ 10⁵ cells/ml; typically the purity was

greater than 95%. Culture media was changed to AIM-V™ media just prior to experimentation with these cells.

Peritoneal macrophages were obtained three separate times each time using one C57BL/6 mouse asphyxiated with CO₂. The peritoneal cavity was rinsed twice with 7 ml ice-cold phosphate-buffered saline (PBS, pH 7.4), and the cell suspension was collected and centrifuged at 800 × g for 5 min. Cells were resuspended with RPMI 1640 medium containing 20% heat-inactivated FBS and antibiotic/antimycotic and incubated in plastic cell culture dishes overnight at 37 °C and 5% CO₂ to allow the macrophages to adhere. The non-adherent peritoneal cells were removed by washing the dishes twice with 37°C PBS. More than 95% of the adherent cells were macrophages as determined by CD11b immunostaining. One day before experiments, cells were plated at a density of 5×10⁵ cells/ml. Culture media was changed to AIM-V™ media just prior to experimentation with these cells.

Primary hippocampal neurons were cultured from embryonic day 16 C57BL/6 mice six separate times each time using fetuses from two pregnant dams. Briefly, the pregnant dams were killed by asphyxiation with CO₂ and the fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto poly-D-lysine-coated glass-bottom 35-mm tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, antibiotic/antimycotic and B27 supplement, and were maintained at 37°C and 5% CO₂ for 11-14 days *in vitro* at which time they were taken for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by morphology and staining for neuronal markers (NeuN, MAP-2).

ELISA assay for TNF-α

TNF-α production was determined by a sandwich ELISA as described previously (Fotheringham et al., 2004) in supernatants of differentiated human U937 and THP-1 cells in the absence or presence of Tat peptides. Briefly, microtiter plates were coated with the primary antibody against human TNF-α and then incubated for 16 h at 4°C with experimental samples. Levels of TNF-α were determined by adding a secondary biotinylated antibody followed by streptavidin-conjugated alkaline phosphatase and plates were developed using phosphatase tablets and read at 405 and 620 nm. To determine the effects of Tat peptides on TNF-α production in primary mouse peritoneal macrophages and brain microglia, supernatants from Tat-treated cells were analyzed with a mouse TNF-α ELISA kit from eBioscience following the instructions provided by the manufacturer.

Tat peptides specificity

To test for specificity, Tat₁₋₇₂ and Tat_{Δ31-61} were immunoabsorbed by adding 1:100 (v:v) dilutions of mouse monoclonal antibody raised against the N-terminal portion of the Tat protein bound to protein G-coated agarose beads. Following incubation for 90 min at room temperature, samples were centrifuged at 14,000 × g for 10 min and supernatants were tested for their ability to increase the release of TNF-α from differentiated U937 cells. To control for the possibility of lipopolysaccharide contamination, separate aliquots of Tat₁₋₇₂ and Tat_{Δ31-61} were heated for 30 minutes at 100°C (lipopolysaccharide is heat-stable) and were tested for their ability to increase the release of TNF-α from differentiated U937 cells. For all experiments, aliquots of Tat peptides were used immediately after thawing, were handled with siliconized tips and tubes, and were not mixed vigorously or refrozen.

Neurotoxicity Assays

The direct effects of Tat peptides on cell viability were determined after 24 h exposure of primary cultures of mouse hippocampal neurons to Tat₁₋₇₂ or Tat_{Δ31-61}. The indirect effects of peptides on neuronal viability were determined after a 24 h exposure of the neuronal cultures

to a 1:4 (v:v) dilution of the media from PMA-differentiated U937 cells or primary macrophages previously incubated with the peptides for 4 h. For immunoneutralization experiments, anti-TNF- α antibody (1 μ g/ml) was added to differentiated U937 cells immediately prior to application of Tat₁₋₇₂, Tat _{Δ 31-61} or vehicle and 4 h later media from these cells was applied to neurons for 24 h.

Neuronal cell viability was determined by triple-staining with 10 μ g/ml Hoechst 33342 (blue fluorescence, excitation/emission wavelengths 346/460 nm), 4 μ M ethidium homodimer-1 (red fluorescence, excitation/emission wavelengths 528/617 nm) and 1 μ g/ml calcein (green fluorescence, excitation/emission wavelengths 494/517 nm). Hoechst 33342, which labels DNA, was used as a marker for identifying condensed nuclei characteristic of apoptotic cell death. Cells dead or dying as a result of loss of membrane integrity were unable to exclude ethidium homodimer dye. Cells were considered viable when cytoplasm was stained with green fluorescence after the cleavage of the non-fluorescent calcein acetoxymethyl ester to calcein. Fields were chosen at random and at least five images of every experimental condition were taken with an Axiovert 200M fluorescence microscope (Zeiss, Jena, Germany). The number of dead or dying neurons (ethidium-labeled red nuclei and blue-condensed nuclei without green cytoplasmic staining) and total neuron numbers were counted using Axiovision software (Zeiss, Jena, Germany). Our cultures were >95% neurons and only cells identified morphologically as neurons were included in our results. More than 300 neurons were counted per dish. Neuronal viability was reported as a percentage of total neurons present and data were analyzed relative to controls.

Statistical analysis

Means and standard errors of the mean were reported throughout the manuscript as well as the number of times each experiment was performed (n). Statistical comparisons were conducted using one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison *post hoc* test. Statistical significance was considered at $p < 0.05$.

Results

Effects of Tat on TNF- α release

We first determined the optimal conditions for Tat-induced increases in TNF- α using PMA-differentiated U937 and THP-1 monocytic cell lines. TNF- α levels increased in a concentration and time-dependent manner after application of Tat₁₋₇₂ at concentrations ranging from 14 to 700 ng/ml; near maximal increases in release were noted at 4 h of treatment for U937 (Fig. 1A) and THP-1 (Fig. 1C) cells. Using 4 h incubations, we next applied concentrations of Tat₁₋₇₂ and Tat _{Δ 31-61} ranging from 1.4 to 4700 ng/ml to compare concentration-dependent increases of TNF- α levels in U937 cells (Fig. 1B), THP-1 (Fig. 1D) cells, peripheral macrophages (Fig. 2E) and brain microglia (Fig. 2F). In all cases, Tat _{Δ 31-61} was as efficacious in increasing TNF- α release as was Tat₁₋₇₂. Primary cultures of macrophages and microglia, the purity of which was confirmed by immunostaining for a monocyte/macrophage lineage marker CD11b (Fig. 2A-D), were used to confirm the data obtained from the established monocytic cell lines because they are the major source of TNF- α *in vivo*. Taking into consideration the lower plating densities of macrophages and microglia for these studies, the TNF- α levels for macrophages (Fig. 2E) and microglia (Fig. 2F) were comparable to the levels from PMA-differentiated U937 (Fig. 1B) and THP-1 (Fig. 1D) cells.

Although the primary cultures of primary macrophages and microglia used were highly purified, we could not exclude the possibility of other contaminating cell types in these cultures. Hence, we used differentiated human U937 cells with incubation periods of 4 h and concentrations of Tat₁₋₇₂ and Tat _{Δ 31-61} of 1.4 μ g/ml for the remaining experiments.

Specificity of Tat₁₋₇₂ - and Tat_{Δ31-61}-induced increases in TNF-α

To confirm the specificity of the actions of Tat₁₋₇₂ and Tat_{Δ31-61}, samples of Tat₁₋₇₂ and Tat_{Δ31-61} were heated for 30 min at 100°C or were immunoadsorbed with a monoclonal antibody directed against the N-terminal region of the Tat protein shared by both peptides. As expected, levels of TNF-α released from differentiated U937 cells were significantly increased when 1.4 μg/ml of Tat₁₋₇₂ (Fig. 3A) or Tat_{Δ31-61} (Fig. 3B) were applied for 4 h. Heating Tat at 100°C for 30 min and immunoadsorption both abolished the ability of Tat₁₋₇₂ (Fig. 3A) and Tat_{Δ31-61} (Fig. 3B) to increase levels of TNF-α. Together, these data indicate that the responses observed were caused by Tat specifically and not due to, for example, lipopolysaccharide that is heat-stable and not susceptible to immunoneutralization by the Tat antibody.

Direct toxicity of Tat₁₋₇₂ and Tat_{Δ31-61}

The viability of primary cultures of hippocampal neurons as determined by triple staining with Hoechst 33342, calcein, and ethidium homodimer was decreased significantly ($p < 0.05$) from control levels of 100 ± 5.0 to 86.6 ± 4.6 % of control levels when Tat₁₋₇₂ was added for 24 h at a concentration of 1.4 μg/ml (Fig. 4). As expected, neuronal viability was not affected significantly when neurons were incubated with 1.4 μg/ml Tat_{Δ31-61} for 24 h (Fig. 4).

Tat₁₋₇₂ and Tat_{Δ31-61} enhanced the neurotoxicity of supernatants from differentiated U937 cells and primary macrophages

To ascertain whether the substances released from Tat-treated U937 cells were neurotoxic, we determined the viability of primary cultures of hippocampal neurons 24 h after incubation with supernatants from U937 cells (Fig. 5) and primary macrophages. Neuronal viability was decreased significantly ($p < 0.001$) to 34.2 ± 4.0 % of control levels when supernatants from vehicle-treated U937 cells were added at a dilution of 1:4 (v:v) to hippocampal neurons. Supernatants from Tat₁₋₇₂ and Tat_{Δ31-61} treated U937 cells were significantly ($p < 0.01$) more neurotoxic than was supernatant from vehicle-treated controls (Fig. 5). Neuronal viability was decreased significantly ($p < 0.01$) by supernatants from Tat₁₋₇₂-treated U937 cells (15.5 ± 3.1 % of control levels) and by supernatants from Tat_{Δ31-61}-treated U937 cells (16.3 ± 1.7 % of control levels). When expressed as a percent of neuronal viability values obtained for neurons treated with media from vehicle-treated U937 cells, neuronal viability was decreased to 50.8 ± 10.2 % by Tat₁₋₇₂ and to 52.5 ± 4.8 % by Tat_{Δ31-61}. To confirm these results, we repeated the above studies with supernatants from primary macrophages. Neuronal viability was decreased significantly ($p < 0.001$) to 44.0 ± 3.5 % of control levels when supernatants from vehicle-treated primary macrophages cells were added at a dilution of 1:4 (v:v) to hippocampal neurons ($n=3$, data not shown). Similar to supernatants from U937 cells, when expressed as a percent of neuronal viability values obtained for neurons treated with media from vehicle-treated primary macrophages, supernatants from Tat₁₋₇₂ and Tat_{Δ31-61} treated primary macrophages produced significantly ($p < 0.05$) more neurotoxicity than did supernatants from vehicle-treated primary macrophages; neuronal viability was decreased to 30.0 ± 8.2 % by Tat₁₋₇₂ and 29.0 ± 7 % by Tat_{Δ31-61} ($n=3$, data not shown).

The role of TNF-α in neurotoxicity caused by supernatants from U937 cells

We next tested the extent to which TNF-α produced by Tat-treated U937 cells could negatively affect neuronal viability (Fig. 6). Differentiated U937 cells were incubated with neutralizing anti-TNF-α antibody immediately prior to application of Tat₁₋₇₂, Tat_{Δ31-61} or media (control), and the supernatants from these cells were applied for 24 h to cultured hippocampal neurons. Pre-incubation with the neutralizing antibody against TNF-α protected against the neuronal cell death induced by supernatants from Tat₁₋₇₂- and Tat_{Δ31-61}-treated U937 cells to a level similar to that observed for supernatants of cells not treated with Tat peptides (Fig. 6). We observed that supernatants from media-treated U937 cells were neurotoxic, but that the

immunoneutralization of TNF- α offered no protection thus indicating that the neurotoxicity was independent of TNF- α . However this neurotoxicity was completely abolished with the pre-treatment of hippocampal neurons with the non-competitive NMDA receptor antagonist dizocilpine (data not shown).

Discussion

The transactivating HIV-1 protein Tat is thought to play an important role in the pathogenesis of HIV-1 associated dementia in part because of findings that Tat has been detected in brains of people with HIV-dementia (Hudson et al., 2000), is actively released from HIV-infected lymphoid and glial cells (Ensoli et al., 1990), can cause neuroinflammation (Philippon et al., 1994; Jones et al., 1998; Aksenov et al., 2003; Pu et al., 2003), and can cause neuronal cell death through direct and indirect mechanisms. In addition to the direct toxic effects of Tat on neurons, Tat promotes the release of neurotoxic agents, including TNF- α , from macrophages and glial cells (Philippon et al., 1994; Chen et al., 1997; Nath et al., 1999; Mayne et al., 2000; Bruce-Keller et al., 2001; Johnston et al., 2001; Bennasser et al., 2002). Here, we found that Tat₁₋₇₂ caused neuronal cell death through increased production of substances by immune cells independently of its direct neurotoxic actions on neurons, and that Tat Δ ₃₁₋₆₁, a mutant Tat peptide lacking the neurotoxic domain that does not kill neurons when applied directly to them, elicited the release of similarly-regulated toxic substances from immune cells which can then kill neurons.

Others and we have shown previously that Tat₁₋₇₂ is neurotoxic (New et al., 1997; Haughey et al., 1999; Gurwell et al., 2001; Prendergast et al., 2002; Self et al., 2004b) and that the amino acid sequence 31-61 includes the neurotoxic epitope (Nath et al., 1996). Tat Δ ₃₁₋₆₁ has been widely used as a negative control for Tat-induced neurotoxicity because it does not decrease neuronal cell viability when added directly to cultured neurons or hippocampal slices (Gurwell et al., 2001; Prendergast et al., 2002; Self et al., 2004a; Aksenov et al., 2006). However, here we showed that Tat Δ ₃₁₋₆₁ was able to induce the release of neurotoxins from monocytic cell lines as well as from highly purified primary cultures of macrophages and microglia. The neurotoxicity of the supernatants could not be attributed to residual Tat in the media because Tat Δ ₃₁₋₆₁ is not directly neurotoxic, and the degree of neuronal cell death caused by supernatants of Tat₁₋₇₂-treated cells was higher than was the neuronal cell death observed with four-times higher concentrations of Tat₁₋₇₂ applied directly to neurons. Furthermore, in preliminary experiments where the supernatants obtained from the Tat₁₋₇₂- and Tat Δ ₃₁₋₆₁-treated U937 cells were heated at 100°C for 60 min we observed no decrease in the toxicity of the heated compared with fresh supernatants (data not shown). These preliminary findings suggest that the toxin in the supernatant was not residual Tat and was not proteinaceous.

TNF- α was an essential factor for the neurotoxicity caused by supernatants of Tat-treated U937 cells because immunoneutralization of TNF- α blocked the supernatant-induced neurotoxicity. This confirms and extends previous findings that Tat-induced neuronal apoptosis was reduced with the use of anti-TNF- α neutralizing antibodies as well as suggestions that macrophages and microglia present in primary cultures of human fetal cortical neurons were the source of the TNF- α (New et al., 1998). Our findings are also consistent with findings that pentoxifylline, an inhibitor of TNF- α synthesis, protected against inflammation and cell loss observed with Tat infusions into mouse brain (Philippon et al., 1994), that in a murine model of HIV-1 encephalitis a TNF- α release inhibitor markedly reduced brain inflammation and neuronal injury (Persidsky et al., 2001), and that gene delivery of soluble tumor necrosis factor receptor protected neurons from Tat-induced cell death (Williams et al., 2005).

The levels of TNF- α produced in response to Tat₁₋₇₂ and Tat Δ ₃₁₋₆₁ were comparable in all four cell types used; the values observed in the primary cultures were lower than the levels in the

differentiated cell lines because fewer cells were used in those experiments. Tat₁₋₇₂ and Tat_{Δ31-61} induced concentration-dependent increases in TNF- α were specific because their effects were blocked when peptides were either boiled (lipopolysaccharide is heat-stable) or immunoadsorbed with anti-Tat antibody. The neurotoxicity that we observed with supernatants from media-treated (control) U937 cells was independent of TNF- α because immunoneutralization of TNF- α in those supernatants did not protect against the neurotoxicity, but was probably due to glutamate in the AIM-V™ medium because the non-competitive antagonist of NMDA receptors, dizocilpine, blocked the neurotoxic effects of the supernatants.

The N-terminal region of Tat has been identified previously as the one that contains the active domain for TNF- α production in macrophages (Bennasser et al., 2002). In a further attempt to identify if a shorter Tat epitope was responsible for the effects on TNF- α release, we added 15-mer peptides that spanned the full-length of Tat and that overlapped by 5 amino acids to differentiated U937 cells at concentrations up to 1 μ M. In contrast to Tat₁₋₇₂ or Tat_{Δ31-61}, none of the Tat 15-mer peptides even at such high concentrations significantly affected levels of TNF- α released from U937 cells (data not shown). Tat-induced TNF- α production seems to be a membrane-triggered event because Tat₁₋₇₂ is poorly internalized (Ma and Nath, 1997), increasing concentrations of immobilized Tat in culture wells caused dose-dependent increases in production of TNF- α (Bennasser et al., 2002), and Tat_{Δ31-61} is able to elicit an increase of TNF- α similar to that of Tat₁₋₇₂, even though amino acids 37-57 are necessary for the binding and uptake of Tat (Liu et al., 2000). Together, these findings suggest that the Tat-induced TNF- α production is mediated through cell-surface interactions, and that the region involved includes the N-terminal domain and spans more than 15 amino acids.

TNF- α by itself was not toxic when added directly to hippocampal neurons at concentrations higher than those present in the supernatants (data not shown), but TNF- α can act as an autocrine factor and can potentiate the toxicity induced by other excitotoxins. Some of the neurotoxins produced from macrophages and/or microglia that can potentially contribute to HIV-related neuronal damage caused by TNF- α and/or Tat and that are elevated in the brains or cerebrospinal fluid of patients living with HIV-dementia include quinolinic acid (Achim et al., 1993; Sei et al., 1995; Pemberton et al., 1997; Smith et al., 2001), platelet-activated factor (Genis et al., 1992; Gelbard et al., 1994; Jaranowska et al., 1995; Westmoreland et al., 1996), and nitric oxide (Bukrinsky et al., 1995; Adamson et al., 1999; Polazzi et al., 1999; Wanchu et al., 2000; Bruce-Keller et al., 2001). Some of these factors may account for observations that supernatants from Tat-treated macrophages caused neurotoxicity to an extent greater than did direct application of Tat onto neurons (Brana et al., 1999).

Development and progression of HIV-associated dementia is thought to be a consequence of immune activation and cytokine dysregulation rather than high viral burden in the CNS. Our data show that, in addition to the direct neurotoxic effect of Tat on neurons, there is a Tat-mediated neuronal cell death independent of the neurotoxic domain of Tat, based on the secretion of neurotoxic substances from immune cells. The indirect neurotoxic effects of Tat occur at lower concentrations of Tat than are required to produce neurotoxicity when applied directly to neurons. Given that concentrations of Tat in the serum and brains of HIV-infected patients are in the low ng/ml range (Westendorp et al., 1995; Hudson et al., 2000; Xiao et al., 2000), and that even a transient exposure of Tat induces a rapid and sustained production of cytokines (Nath et al., 1999), the indirect mechanism described here might be the predominant neurotoxic mechanism. Thus, even very low concentrations of viral proteins can lead to inflammatory responses with an important associated neurotoxicity, and the use of anti-inflammatory agents aimed at lowering TNF- α levels such as minocycline (Zink et al., 2005) and adenosine receptor agonists (Fotheringham et al., 2004) might be useful therapeutic strategies for prevention/treatment of HIV-1 associated dementia.

Acknowledgements

This work was supported by grants from the NCRR (P20 RR17699) a component of the NIH, the National Institute of Aging (AG17628), and the Canadian Institutes of Health Research (HOP-8901, MOP-53329). The authors would like to thank Dr. Avindra Nath for supplying us with Tat.

Bibliography

- Achim CL, Heyes MP, Wiley CA. Quantitation of human immunodeficiency virus, immune activation factors, and quinolinic acid in AIDS brains. *J Clin Invest* 1993;91:2769–2775. [PubMed: 8514884]
- Adamson DC, McArthur JC, Dawson TM, Dawson VL. Rate and severity of HIV-associated dementia (HAD): correlations with Gp41 and iNOS. *Mol Med* 1999;5:98–109. [PubMed: 10203575]
- Aksenov MY, Hasselrot U, Wu G, Nath A, Anderson C, Mactutus CF, Booze RM. Temporal relationships between HIV-1 Tat-induced neuronal degeneration, OX-42 immunoreactivity, reactive astrocytosis, and protein oxidation in the rat striatum. *Brain Res* 2003;987:1–9. [PubMed: 14499939]
- Aksenov MY, Aksenova MV, Nath A, Ray PD, Mactutus CF, Booze RM. Cocaine-mediated enhancement of Tat toxicity in rat hippocampal cell cultures: the role of oxidative stress and D1 dopamine receptor. *Neurotoxicology* 2006;27:217–228. [PubMed: 16386305]
- Bennasser Y, Badou A, Tkaczuk J, Bahraoui E. Signaling pathways triggered by HIV-1 Tat in human monocytes to induce TNF-alpha. *Virology* 2002;303:174–180. [PubMed: 12482669]
- Bonavia R, Bajetto A, Barbero S, Albini A, Noonan DM, Schettini G. HIV-1 Tat causes apoptotic death and calcium homeostasis alterations in rat neurons. *Biochem Biophys Res Commun* 2001;288:301–308. [PubMed: 11606043]
- Bouwman FH, Skolasky RL, Hes D, Selnes OA, Glass JD, Nance-Sproson TE, Royal W, Dal Pan GJ, McArthur JC. Variable progression of HIV-associated dementia. *Neurology* 1998;50:1814–1820. [PubMed: 9633733]
- Brana C, Biggs TE, Barton CH, Sundstrom LE, Mann DA. A soluble factor produced by macrophages mediates the neurotoxic effects of HIV-1 Tat in vitro. *Aids* 1999;13:1443–1452. [PubMed: 10465066]
- Brew BJ, Rosenblum M, Cronin K, Price RW. AIDS dementia complex and HIV-1 brain infection: clinical-virological correlations. *Ann Neurol* 1995;38:563–570. [PubMed: 7574452]
- Bruce-Keller AJ, Barger SW, Moss NI, Pham JT, Keller JN, Nath A. Pro-inflammatory and pro-oxidant properties of the HIV protein Tat in a microglial cell line: attenuation by 17 beta-estradiol. *J Neurochem* 2001;78:1315–1324. [PubMed: 11579140]
- Bukrinsky MI, Nottet HS, Schmidtmayerova H, Dubrovsky L, Flanagan CR, Mullins ME, Lipton SA, Gendelman HE. Regulation of nitric oxide synthase activity in human immunodeficiency virus type 1 (HIV-1)-infected monocytes: implications for HIV-associated neurological disease. *J Exp Med* 1995;181:735–745. [PubMed: 7530762]
- Buonaguro L, Barillari G, Chang HK, Bohan CA, Kao V, Morgan R, Gallo RC, Ensoli B. Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. *J Virol* 1992;66:7159–7167. [PubMed: 1279199]
- Chen P, Mayne M, Power C, Nath A. The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. *J Biol Chem* 1997;272:22385–22388. [PubMed: 9278385]
- Cheng J, Nath A, Knudsen B, Hochman S, Geiger JD, Ma M, Magnuson DS. Neuronal excitatory properties of human immunodeficiency virus type 1 Tat protein. *Neuroscience* 1998;82:97–106. [PubMed: 9483506]
- Ellis RJ, Hsia K, Spector SA, Nelson JA, Heaton RK, Wallace MR, Abramson I, Atkinson JH, Grant I, McCutchan JA. Cerebrospinal fluid human immunodeficiency virus type 1 RNA levels are elevated in neurocognitively impaired individuals with acquired immunodeficiency syndrome. HIV Neurobehavioral Research Center Group. *Ann Neurol* 1997;42:679–688. [PubMed: 9392566]
- Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfield P, Gallo RC. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J Virol* 1993;67:277–287. [PubMed: 8416373]

- Ensoli B, Barillari G, Salahuddin SZ, Gallo RC, Wong-Staal F. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. *Nature* 1990;345:84–86. [PubMed: 2184372]
- Flora G, Pu H, Lee YW, Ravikumar R, Nath A, Hennig B, Toborek M. Proinflammatory synergism of ethanol and HIV-1 Tat protein in brain tissue. *Exp Neurol* 2005;191:2–12. [PubMed: 15589507]
- Fotheringham J, Mayne M, Holden C, Nath A, Geiger JD. Adenosine receptors control HIV-1 Tat-induced inflammatory responses through protein phosphatase. *Virology* 2004;327:186–195. [PubMed: 15351206]
- Gelbard HA, Nottet HS, Swindells S, Jett M, Dzenko KA, Genis P, White R, Wang L, Choi YB, Zhang D, et al. Platelet-activating factor: a candidate human immunodeficiency virus type 1-induced neurotoxin. *J Virol* 1994;68:4628–4635. [PubMed: 8207837]
- Genis P, Jett M, Bernton EW, Boyle T, Gelbard HA, Dzenko K, Keane RW, Resnick L, Mizrachi Y, Volsky DJ, et al. Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. *J Exp Med* 1992;176:1703–1718. [PubMed: 1460427]
- Glass JD, Wesselingh SL. Microglia in HIV-associated neurological diseases. *Microsc Res Tech* 2001;54:95–105. [PubMed: 11455616]
- Glass JD, Fedor H, Wesselingh SL, McArthur JC. Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. *Ann Neurol* 1995;38:755–762. [PubMed: 7486867]
- Glass JD, Wesselingh SL, Selnes OA, McArthur JC. Clinical-neuropathologic correlation in HIV-associated dementia. *Neurology* 1993;43:2230–2237. [PubMed: 8232935]
- Grimaldi LM, Martino GV, Franciotta DM, Brustia R, Castagna A, Pristera R, Lazzarin A. Elevated alpha-tumor necrosis factor levels in spinal fluid from HIV-1-infected patients with central nervous system involvement. *Ann Neurol* 1991;29:21–25. [PubMed: 1996875]
- Gurwell JA, Nath A, Sun Q, Zhang J, Martin KM, Chen Y, Hauser KF. Synergistic neurotoxicity of opioids and human immunodeficiency virus-1 Tat protein in striatal neurons in vitro. *Neuroscience* 2001;102:555–563. [PubMed: 11226693]
- Haughey NJ, Holden CP, Nath A, Geiger JD. Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calcium in calcium dysregulation and neuron cell death caused by HIV-1 protein tat. *J Neurochem* 1999;73:1363–1374. [PubMed: 10501179]
- Hudson L, Liu J, Nath A, Jones M, Raghavan R, Narayan O, Male D, Everall I. Detection of the human immunodeficiency virus regulatory protein tat in CNS tissues. *J Neurovirol* 2000;6:145–155. [PubMed: 10822328]
- Jaranowska A, Bussolino F, Sogos V, Arese M, Lauro GM, Gremo F. Platelet-activating factor production by human fetal microglia. Effect of lipopolysaccharides and tumor necrosis factor-alpha. *Mol Chem Neuropathol* 1995;24:95–106. [PubMed: 7632332]
- Johnston JB, Zhang K, Silva C, Shalinsky DR, Conant K, Ni W, Corbett D, Yong VW, Power C. HIV-1 Tat neurotoxicity is prevented by matrix metalloproteinase inhibitors. *Ann Neurol* 2001;49:230–241. [PubMed: 11220743]
- Jones M, Olafson K, Del Bigio MR, Peeling J, Nath A. Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement. *J Neuropathol Exp Neurol* 1998;57:563–570. [PubMed: 9630236]
- Kaul M, Zheng J, Okamoto S, Gendelman HE, Lipton SA. HIV-1 infection and AIDS: consequences for the central nervous system. *Cell Death Differ* 2005;12(Suppl 1):878–892. [PubMed: 15832177]
- Kruman, Nath A, Mattson MP. HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism involving caspase activation, calcium overload, and oxidative stress. *Exp Neurol* 1998;154:276–288. [PubMed: 9878167]
- Liu Y, Jones M, Hingtgen CM, Bu G, Larabee N, Tanzi RE, Moir RD, Nath A, He JJ. Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. *Nat Med* 2000;6:1380–1387. [PubMed: 11100124]
- Ma M, Nath A. Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. *J Virol* 1997;71:2495–2499. [PubMed: 9032389]

- Magnuson DS, Knudsen BE, Geiger JD, Brownstone RM, Nath A. Human immunodeficiency virus type 1 tat activates non-N-methyl-D-aspartate excitatory amino acid receptors and causes neurotoxicity. *Ann Neurol* 1995;37:373–380. [PubMed: 7695237]
- Maragos WF, Young KL, Turchan JT, Guseva M, Pauly JR, Nath A, Cass WA. Human immunodeficiency virus-1 Tat protein and methamphetamine interact synergistically to impair striatal dopaminergic function. *J Neurochem* 2002;83:955–963. [PubMed: 12421368]
- Mayne M, Holden CP, Nath A, Geiger JD. Release of calcium from inositol 1,4,5-trisphosphate receptor-regulated stores by HIV-1 Tat regulates TNF-alpha production in human macrophages. *J Immunol* 2000;164:6538–6542. [PubMed: 10843712]
- McArthur JC. HIV dementia: an evolving disease. *J Neuroimmunol* 2004;157:3–10. [PubMed: 15579274]
- Nath A, Berger J. HIV Dementia. *Curr Treat Options Neurol* 2004;6:139–151. [PubMed: 14759346]
- Nath A, Psooy K, Martin C, Knudsen B, Magnuson DS, Haughey N, Geiger JD. Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. *J Virol* 1996;70:1475–1480. [PubMed: 8627665]
- Nath A, Geiger J. Neurobiological aspects of human immunodeficiency virus infection: neurotoxic mechanisms. *Prog Neurobiol* 1998;54:19–33. [PubMed: 9460791]
- Nath A, Conant K, Chen P, Scott C, Major EO. Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem* 1999;274:17098–17102. [PubMed: 10358063]
- New DR, Ma M, Epstein LG, Nath A, Gelbard HA. Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. *J Neurovirol* 1997;3:168–173. [PubMed: 9111179]
- New DR, Maggirwar SB, Epstein LG, Dewhurst S, Gelbard HA. HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB-independent mechanism. *J Biol Chem* 1998;273:17852–17858. [PubMed: 9651389]
- Pemberton LA, Kerr SJ, Smythe G, Brew BJ. Quinolinic acid production by macrophages stimulated with IFN-gamma, TNF-alpha, and IFN-alpha. *J Interferon Cytokine Res* 1997;17:589–595. [PubMed: 9355959]
- Perry SW, Norman JP, Litzburg A, Zhang D, Dewhurst S, Gelbard HA. HIV-1 transactivator of transcription protein induces mitochondrial hyperpolarization and synaptic stress leading to apoptosis. *J Immunol* 2005;174:4333–4344. [PubMed: 15778398]
- Persidsky Y, Limoges J, Rasmussen J, Zheng J, Gearing A, Gendelman HE. Reduction in glial immunity and neuropathology by a PAF antagonist and an MMP and TNFalpha inhibitor in SCID mice with HIV-1 encephalitis. *J Neuroimmunol* 2001;114:57–68. [PubMed: 11240016]
- Philippon V, Vellutini C, Gambarelli D, Harkiss G, Arbuthnott G, Metzger D, Roubin R, Filippi P. The basic domain of the lentiviral Tat protein is responsible for damages in mouse brain: involvement of cytokines. *Virology* 1994;205:519–529. [PubMed: 7526541]
- Polazzi E, Levi G, Minghetti L. Human immunodeficiency virus type 1 Tat protein stimulates inducible nitric oxide synthase expression and nitric oxide production in microglial cultures. *J Neuropathol Exp Neurol* 1999;58:825–831. [PubMed: 10446807]
- Prendergast MA, Rogers DT, Mulholland PJ, Littleton JM, Wilkins LH Jr, Self RL, Nath A. Neurotoxic effects of the human immunodeficiency virus type-1 transcription factor Tat require function of a polyamine sensitive-site on the N-methyl-D-aspartate receptor. *Brain Res* 2002;954:300–307. [PubMed: 12414113]
- Pu H, Tian J, Flora G, Lee YW, Nath A, Hennig B, Toborek M. HIV-1 Tat protein upregulates inflammatory mediators and induces monocyte invasion into the brain. *Mol Cell Neurosci* 2003;24:224–237. [PubMed: 14550782]
- Rappaport J, Joseph J, Croul S, Alexander G, Del Valle L, Amini S, Khalili K. Molecular pathway involved in HIV-1-induced CNS pathology: role of viral regulatory protein, Tat. *J Leukoc Biol* 1999;65:458–465. [PubMed: 10204574]
- Sabatier JM, Vives E, Mabrouk K, Benjouad A, Rochat H, Duval A, Hue B, Bahraoui E. Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J Virol* 1991;65:961–967. [PubMed: 1898974]

- Sacktor N, McDermott MP, Marder K, Schifitto G, Selnes OA, McArthur JC, Stern Y, Albert S, Palumbo D, Kieburtz K, De Marcaida JA, Cohen B, Epstein L. HIV-associated cognitive impairment before and after the advent of combination therapy. *J Neurovirol* 2002;8:136–142. [PubMed: 11935465]
- Saha RN, Pahan K. Tumor necrosis factor- α at the crossroads of neuronal life and death during HIV-associated dementia. *J Neurochem* 2003;86:1057–1071. [PubMed: 12911614]
- Saura J, Tusell JM, Serratos J. High-yield isolation of murine microglia by mild trypsinization. *Glia* 2003;44:183–189. [PubMed: 14603460]
- Sawaya BE, Thatikunta P, Denisova L, Brady J, Khalili K, Amini S. Regulation of TNF α and TGF β -1 gene transcription by HIV-1 Tat in CNS cells. *J Neuroimmunol* 1998;87:33–42. [PubMed: 9670843]
- Sei S, Saito K, Stewart SK, Crowley JS, Brouwers P, Kleiner DE, Katz DA, Pizzo PA, Heyes MP. Increased human immunodeficiency virus (HIV) type 1 DNA content and quinolinic acid concentration in brain tissues from patients with HIV encephalopathy. *J Infect Dis* 1995;172:638–647. [PubMed: 7658054]
- Self RL, Mulholland PJ, Harris BR, Nath A, Prendergast MA. Cytotoxic effects of exposure to the human immunodeficiency virus type 1 protein Tat in the hippocampus are enhanced by prior ethanol treatment. *Alcohol Clin Exp Res* 2004a;28:1916–1924. [PubMed: 15608609]
- Self RL, Mulholland PJ, Nath A, Harris BR, Prendergast MA. The human immunodeficiency virus type-1 transcription factor Tat produces elevations in intracellular Ca²⁺ that require function of an N-methyl-D-aspartate receptor polyamine-sensitive site. *Brain Res* 2004b;995:39–45. [PubMed: 14644469]
- Sevigny JJ, Albert SM, McDermott MP, McArthur JC, Sacktor N, Conant K, Schifitto G, Selnes OA, Stern Y, McClernon DR, Palumbo D, Kieburtz K, Riggs G, Cohen B, Epstein LG, Marder K. Evaluation of HIV RNA and markers of immune activation as predictors of HIV-associated dementia. *Neurology* 2004;63:2084–2090. [PubMed: 15596754]
- Shi B, Raina J, Lorenzo A, Busciglio J, Gabuzda D. Neuronal apoptosis induced by HIV-1 Tat protein and TNF- α : potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia. *J Neurovirol* 1998;4:281–290. [PubMed: 9639071]
- Smith DG, Guillemain GJ, Pemberton L, Kerr S, Nath A, Smythe GA, Brew BJ. Quinolinic acid is produced by macrophages stimulated by platelet activating factor, Nef and Tat. *J Neurovirol* 2001;7:56–60. [PubMed: 11519483]
- Takahashi K, Wesselingh SL, Griffin DE, McArthur JC, Johnson RT, Glass JD. Localization of HIV-1 in human brain using polymerase chain reaction/in situ hybridization and immunocytochemistry. *Ann Neurol* 1996;39:705–711. [PubMed: 8651642]
- Toborek M, Lee YW, Pu H, Malecki A, Flora G, Garrido R, Hennig B, Bauer HC, Nath A. HIV-Tat protein induces oxidative and inflammatory pathways in brain endothelium. *J Neurochem* 2003;84:169–179. [PubMed: 12485413]
- Tyor WR, Glass JD, Griffin JW, Becker PS, McArthur JC, Bezman L, Griffin DE. Cytokine expression in the brain during the acquired immunodeficiency syndrome. *Ann Neurol* 1992;31:349–360. [PubMed: 1586135]
- Wanchu A, Khullar M, Bhatnagar A, Sud A, Bamberg P, Singh S. Pentoxifylline reduces nitric oxide production among patients with HIV infection. *Immunol Lett* 2000;74:121–125. [PubMed: 10996387]
- Wesselingh SL, Takahashi K, Glass JD, McArthur JC, Griffin JW, Griffin DE. Cellular localization of tumor necrosis factor mRNA in neurological tissue from HIV-infected patients by combined reverse transcriptase/polymerase chain reaction in situ hybridization and immunohistochemistry. *J Neuroimmunol* 1997;74:1–8. [PubMed: 9119960]
- Wesselingh SL, Power C, Glass JD, Tyor WR, McArthur JC, Farber JM, Griffin JW, Griffin DE. Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. *Ann Neurol* 1993;33:576–582. [PubMed: 8498837]
- Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* 1995;375:497–500. [PubMed: 7539892]

- Westmoreland SV, Kolson D, Gonzalez-Scarano F. Toxicity of TNF alpha and platelet activating factor for human NT2N neurons: a tissue culture model for human immunodeficiency virus dementia. *J Neurovirol* 1996;2:118–126. [PubMed: 8799203]
- Wiley CA, Soontornniyomkij V, Radhakrishnan L, Masliah E, Mellors J, Hermann SA, Dailey P, Achim CL. Distribution of brain HIV load in AIDS. *Brain Pathol* 1998;8:277–284. [PubMed: 9546286]
- Williams MA, Turchan J, Lu Y, Nath A, Drachman DB. Protection of human cerebral neurons from neurodegenerative insults by gene delivery of soluble tumor necrosis factor p75 receptor. *Exp Brain Res* 2005;165:383–391. [PubMed: 15827736]
- Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, Jeang KT. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc Natl Acad Sci U S A* 2000;97:11466–11471. [PubMed: 11027346]
- Zink MC, Uhrlaub J, DeWitt J, Voelker T, Bullock B, Mankowski J, Tarwater P, Clements J, Barber S. Neuroprotective and anti-human immunodeficiency virus activity of minocycline. *Jama* 2005;293:2003–2011. [PubMed: 15855434]

Figure 1.

Tat-induced TNF- α release in differentiated monocytes was time and concentration-dependent. Tat₁₋₇₂ (● 14 ng/ml; ■ 70 ng/ml; ◆ 140 ng/ml; ▲ 700 ng/ml) induced a rapid concentration-dependent increase in TNF- α from differentiated U937 (A) and THP-1 cells (C). Tat₁₋₇₂ (open squares) and Tat _{Δ 31-61} (open circles) concentration-dependently increased levels of TNF- α from differentiated U937 (B) and THP-1 cells (D). Basal levels of TNF- α for U937 cells were 88.1 ± 8.2 pg/ml and for THP-1 cells 20.3 ± 5.6 pg/ml. Values are means and SEM. Tat₁₋₇₂ time-course (n=3) and concentration-response (n=3) experiments, and Tat _{Δ 31-61} concentration-response (n=5) experiments for each cell line.

Figure 2.

Tat₁₋₇₂ and Tat_{Δ31-61} concentration-dependently increased levels of TNF-α in primary macrophages and microglia. Phase contrast microscopic images of primary macrophages (A) and microglia (C), and immunohistochemical staining for CD11b of macrophages (B) and microglial (D) confirmed the purity of the preparations. Tat₁₋₇₂ (open squares) and Tat_{Δ31-61} (open circles) concentration-dependently (4 h incubations) increased levels of TNF-α from primary macrophages (E) and microglia (F). Basal levels of TNF-α for macrophages were 4.9 ± 1.2 pg/mL and for microglia were 11.0 ± 1.8 pg/mL. Values are means and SEM of experiments conducted three separate times (n=3) each time using different batches of cells.

Figure 3.

Specificity of Tat₁₋₇₂- and Tat_{Δ31-61}-induced increases in levels of TNF- α . Tat₁₋₇₂- (1.4 μ g/ml) and Tat_{Δ31-61}- (1.4 μ g/ml) induced increases in levels of TNF- α from U937 cells were blocked significantly by immunoadsorption with anti-Tat antibody (Ab) and by heating the peptide at 100°C (100°C). For all conditions tested, values are means and SEM (n=4). * p < 0.05 versus Tat₁₋₇₂ (A) or Tat_{Δ31-61} (B).

Figure 4.

Effects of Tat₁₋₇₂ and Tat_{Δ31-61} on neuronal viability when applied directly to neurons. Tat₁₋₇₂ (1.4 μg/ml) but not Tat_{Δ31-61} (1.4 μg/ml) decreased significantly ($p < 0.05$) the viability of hippocampal neurons following 24 h treatments. Values are means and SEM of experiments conducted five separate times ($n=5$) each time using different batches of cells. * $p < 0.05$ versus control.

Figure 5.

Effects of supernatants of Tat₁₋₇₂ and Tat_{Δ31-61} treated U937 cells on neuronal viability. (A) Phase contrast with overlying fluorescence images of primary hippocampal neurons stained with calcein (green), ethidium homodimer (red) and Hoechst 33342 (blue). 24 h after media from U937 cells was added to neurons, viable neurons (arrows) exhibited green fluorescence staining after esterase hydrolysis of calcein-AM. Non-viable cells (arrow heads) exhibited chromatin condensation when stained with Hoechst 33342, were unable to exclude ethidium homodimer, and lacked neuritic processes. Bar, 50 μ m. (B) Media from U937 cells (sup.) and to a significantly greater extent media from U937 cells treated with 1.4 μ g/ml of Tat₁₋₇₂ (Tat sup.) or Tat_{Δ31-61} (mTat sup.) decreased significantly neuronal viability. Neuronal survival was expressed as a percent of control conditions. Values are means and SEM of determinations made using six separate batches of cultured cells (n=6).

Figure 6.

TNF- α immunoneutralization blocked the neurotoxic effects of supernatants from Tat₁₋₇₂ and Tat _{Δ 31-61} treated U937 cells. Immunoneutralization of TNF- α with anti-TNF- α antibody (1 μ g/ml) decreased significantly neuronal cell death caused by supernatants from U937 cells treated with 1.4 μ g/ml Tat₁₋₇₂ (Tat sup. vs. Tat sup. + Ab) and supernatants from U937 cells treated with 1.4 μ g/ml Tat _{Δ 31-61} (mTat sup. vs. mTat sup. + Ab). Neuronal survival was expressed as a percent of values obtained for neurons treated with media from vehicle-treated U937 cells (sup). Values are means and SEM of determinations made using four separate batches of cultured cells (n=4). Bar, 50 μ m. * p < 0.05 sup vs. Tat sup. or mTat sup.