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Intraventricular Injection of Human Immunodeficiency Virus Type 1 (HIV-1) Tat Protein Causes Inflammation, Gliosis, Apoptosis, and Ventricular Enlargement

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Abstract. To determine the role of the Tat protein of the human immunodeficiency virus type 1 (HIV-1) in the pathogenesis of HIV-1 associated dementia, recombinant Tat was injected intraventricularly as a single or repeated dose into male Sprague-Dawley rats. Histopathological evaluation showed an initial infiltration of neutrophils one day after Tat injection, followed by macrophages and lymphocytes by 7 days. Tat-injected brains also exhibited astrocytosis, apoptotic cells, and ventricular enlargement 7 days following the last injection. Nuclear magnetic resonance spectroscopic analysis of tissue extracts of hippocampi from Tat-injected rats showed a decrease in the glutamate/g aminobutyric acid ratio. We conclude that the transient extracellular exposure of the central nervous system to Tat protein of HIV can cause a cascade of events leading to the influx of inflammatory cells, glial cell activation, and neurotoxicity.

Key Words: AIDS; Apoptosis; Brain; Inflammation, HIV-1, NMR, Tat.

INTRODUCTION

Frequently, patients with human immunodeficiency virus type 1 (HIV-1) infection develop a dementing illness (1, 2) accompanied by cerebral atrophy, gliosis, and neuronal loss in subcortical structures and discrete cortical areas. White matter pallor and mild chronic infiltrations of inflammatory cells are also often associated with HIV infection of the central nervous system (3–5).

As HIV-1 only rarely infects neurons, an indirect mechanism of neuronal toxicity is likely at work. It is thus suggested that infected macrophages or microglial cells in the brain release toxic substances that cause neuronal cell loss. Several viral and cellular products have been implicated (reviewed in 6). Their roles may not be mutually exclusive and may be interdependent. Viral products with neurotoxic properties include the envelope protein gp120, which acts primarily by blocking glutamate uptake (7, 8), and the HIV-1 transactivating protein, Tat (9–13). Isolated reports suggest another structural protein, gp41 (14–15), and the 2 regulatory proteins, Nef (16) and Rev (17–18) may also be neurotoxic. However, unlike other HIV proteins, Tat is released extracellularly from unruptured, HIV-infected lymphoid cells and microglial cells (19–21), and hence has the opportunity to interact with intact cells. Tat exits from cells via a leaderless secretory pathway in the absence of permeability changes (22). Further, Tat can be detected in the sera of HIV-infected individuals (23), and Tat-mRNA levels are elevated in the brains of patients with HIV dementia (24, 25).

We have previously shown that Tat causes depolarizations of the neuronal cell membrane, which is mediated by excitatory amino acid receptors and results in increases in intracellular calcium and neuronal cell death (11, 26). The neurotoxic properties of Tat are conformation-dependent (11) and the neurotoxic domain is located within the amino acid residues 31–61 (13). Tat has a number of other biological effects, which include repression of protein kinase R (PKR) (27, 28), major histocompatibility complex (MHC) class I expression (29), manganese (Mn) superoxide dismutase (30), and antigen-induced lymphocyte proliferation (31). Although the mechanisms underlying the varied functions of Tat are poorly understood, they can be broadly classified as those requiring cellular uptake of Tat, such as Tat-induced transactivation (32, 33), and those requiring interaction of Tat with the cellular membrane only (11, 34–37).

Tat is a nonglycosylated 86 to 101 amino acid protein. The first 72 amino acids are derived from the first exon of the Tat gene and the remainder from the second exon. We have recently determined that deletion of the amino acids formed from the second exon prevents cellular uptake without altering its neurotoxic properties (26, 38). Hence, to determine the in vivo relevance of Tat-mediated neurotoxicity and to determine effects of Tat by cell membrane interaction only, we injected recombinant Tat derived from the first exon (i.e. Tat 1–72) into rat brains and determined the histopathological changes.

MATERIALS AND METHODS

Tat Synthesis and Purification

The tat gene encoding amino acids 1 to 72 (first exon) from HIV-1BRU was expressed as a fusion protein with a naturally biotinylated protein at the N-terminus in *Escherichia coli*

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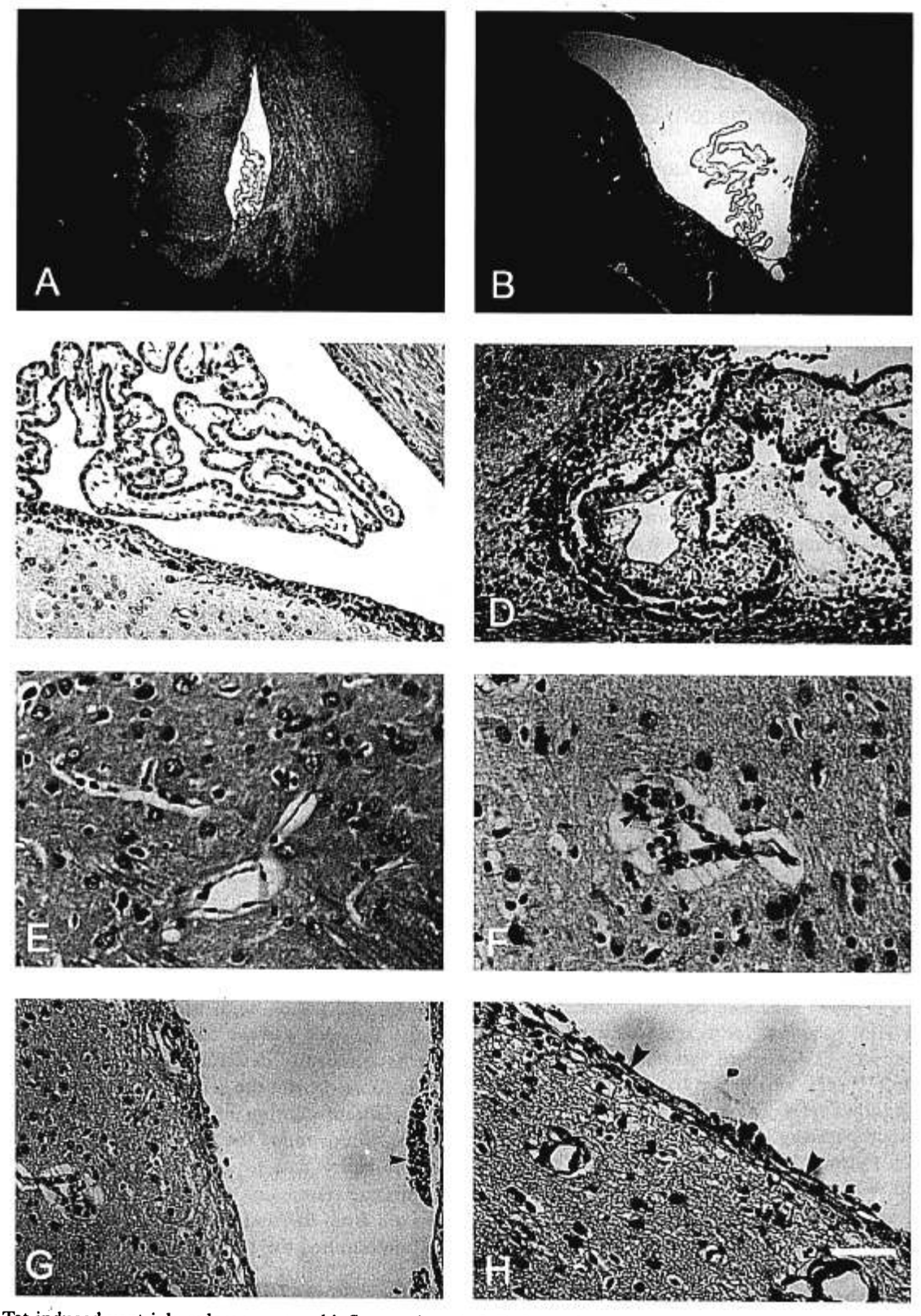

Fig. 1. Tat-induced ventricle enlargement and inflammation. A & B: Lateral ventricle adjacent to the hippocampus and fimbria. Seven days after treatment, the (A) BSA-injected ventricle is of normal size and, (B) the Tat-injected ventricle is enlarged. C & D: One day after injection, the choroid plexus of (C) the BSA-injected rat displays normal morphology while (D) the Tat-injected rat is edematous and infiltrated by inflammatory cells. E & F: Seven days after a single injection, periventricular blood vessels of (E) the BSA-injected rat show no perivascular infiltrates, while (F) the Tat-injected rat shows mononuclear cells in the

DH5αF'IQ (Gibco-BRL). The biotin portion of the fusion protein was first bound to SoftLink® soft-release avidin resin (Promega). Tat protein was then cleaved from the resin with factor Xa, a serine endopeptidase (Boehringer Mannheim). Diothioreitol (DTT) was added in each step of the purification. Tat protein was then desalted. The Tat protein was >95% pure by gel electrophoresis. The purified product was further analyzed by Western immunoblot analysis. Its biological activity was measured by its ability to activate the β-galactosidase (β-gal) gene in an HIV long terminal repeat (LTR)-β-gal plasmid that had been transfected into HeLa cells (AIDS Repository, National Institutes of Health) (38).

Intraventricular Injections

Male Sprague-Dawley (SD) rats weighing 150–220 g were anesthetized with Somnotol (MTC Pharmaceuticals, 28 mg/kg) and placed in a stereotactic frame. Single injections of 5.7×10^{-9} moles of either bovine serum albumin (BSA; Sigma), purified Tat protein, or biotinylated-Tat dissolved in 20 μl of phosphate buffered saline (PBS; pH = 7.4) were delivered by a Hamilton syringe (30 gauge needle) at a rate of 2 μl/minute (min) into the lateral ventricle. Single injections were also performed with a solution from which Tat was immunoeluted by rabbit polyclonal antisera directed against Tat (National Biological Laboratories, Ltd.) conjugated to protein A sepharose beads. Coordinates used for all injections were +0.6 anterior, −0.13 lateral, and −0.44 vertical with respect to the bregma. Rats were killed after 1 day (BSA, n = 2; Tat, n = 3), 3 days (BSA, n = 3; Tat, n = 3), or 7 days (BSA, n = 5; Tat, n = 4; immunoeluted solution, n = 2). For repeated injections, an indwelling cannula (26 gauge) was inserted into the lateral ventricle. One week after surgery, daily injections of 1.14×10^{-9} moles of either Tat or BSA in 20 μl of PBS were performed (BSA, n = 5; Tat, n = 5) for 5 days on each rat. Rats were killed 7 days after the first injection. Animals injected with biotinylated Tat were killed at 30 min (n = 2), 1 hour (n = 3), 6 hours (n = 2), or 22 hours (n = 2).

Immunohistopathology

Rats were anesthetized and perfused through the heart, first with sterile physiological saline (150 mM NaCl) and then with 4% (w/v) paraformaldehyde in PBS. The brains were removed, sliced in the coronal plane, and embedded in paraffin. Six-μm-thick tissue sections were cut and put on gelatin-coated slides. Tissue sections on slides were stained with hematoxylin and eosin (H&E) and solochrome cyanine for myelin. For immunohistochemistry, tissue on slides was dewaxed and rehydrated. Endogenous peroxidases were quenched by immersing the tissue in 1% H_2O_2 in PBS for 30 min. Tissues were blocked with a solution containing 10% horse serum and 0.01% BSA for 30 min at 37°C. Then either of the following primary antibodies were applied: mouse monoclonal anti-GFAP (glial fibrillary acidic protein) antisera (Chemicon; 1:100; for astrocytes) or rabbit polyclonal anti-ferritin antisera (Sigma; 1:5,000, for macrophages). To optimize staining with the anti-ferritin antisera, the tissue sections were microwaved at 800 Watts for 5 min in 0.1 M sodium citrate buffer (pH 6.0) prior to adding the antisera (39). Following application of primary antibody solution, slides were incubated for 90 min at room temperature for anti-GFAP treatment and overnight at 4°C for anti-ferritin treatment. The primary antibody was washed off, and a 1:100 dilution of the appropriate peroxidase-conjugated secondary antibody was applied and incubated for 90 min at room temperature. Immunoreactive complexes were revealed with 0.05% 3, 3′ diaminobenzidine (DAB, Sigma). Biotinylated Tat was localized by streptavidin-conjugated peroxidase (Chemicon).

Labeling of cells undergoing apoptosis was performed using the Apotag kit (Oncor). This procedure labels the ends of DNA fragments produced as a result of cell death with an immune complex such that apoptotic nuclei can be visualized via a peroxidase reaction. The manufacturer's instructions were followed except that the TdT enzyme (terminal deoxynucleotidyl transferase) reagent supplied in the kit was diluted 1:10 instead of the recommended 1:3 to reduce background. For each rat, apoptotic cells were counted in 6-mm-thick coronal sections: one section each from the level of the hippocampus, the basal ganglia, and the frontal lobe. The Mann-Whitney statistical test was performed on this data.

Extraction of Brain Metabolites and Nuclear Magnetic Resonance (NMR) Analysis

Two male SD rats were singly injected with Tat and 3 rats were injected with BSA. Seven days later, these animals were killed using microwave irradiation (10 kWatts, 1.8 s) to achieve enzyme inactivation (40). Injected and uninjected hemispheres were separated and the cortex, basal ganglia, and hippocampus were dissected free. These samples were separately frozen at −70°C, lyophilized for 48 hours, then stored at −70°C until use. Dried tissues were weighed and homogenized in a KHCO3-saturated solution in deuterated water (D2O) (40). Solid material was removed by centrifugation (3 times at 10,000 rpm at 4°C for 30 min), each time retaining the clear middle layer (cellular debris pellet at bottom, lipid layer on top). Final samples were each diluted up to 0.5 ml with D2O. Chemical shift reference (sodium 3- trimethylsylylpropionate 2,2,3,3-D4; TSP) was added to each sample and the resulting solution was placed in a 5 mm NMR tube. NMR spectra were obtained using a Bruker AMX-500 spectrometer. Assignment of peaks to the major metabolites of interest followed directly from previous studies of animal and human tissue (41). Metabolite levels were determined by comparing the integrated intensity of assigned peaks of each compound to that of the creatine + phosphocreatine resonance at 3.93 ppm. Reported mean glutamate: GABA

←

perivascular region (arrowhead) without infiltration of the vessel wall. G: Lateral ventricle of a rat injected once with Tat, showing inflammatory infiltrates (arrowhead) on the ventricle wall; H: periventricular region of a repeatedly Tat-injected rat, showing perivascular infiltrates (small arrowhead) and damaged ependymal lining (large arrows). Bar represents 250 mm in A and B, 50 mm in C, D, and G, and 25 mm in E, F, and H. A–H: H & E.

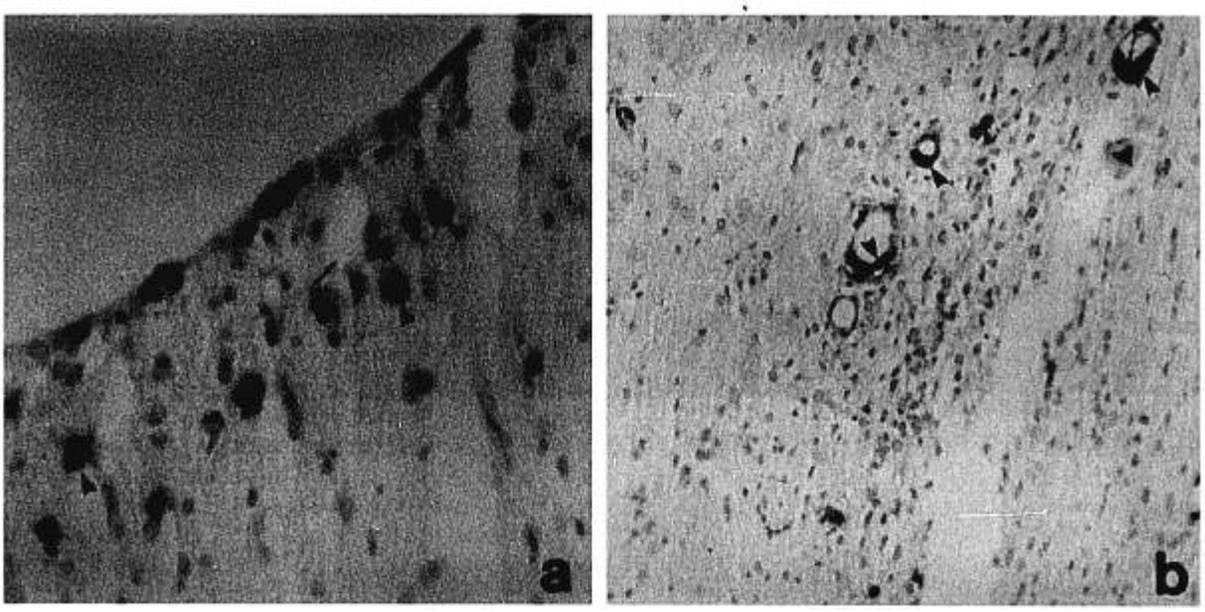

Fig. 2. Tat-induced ferritin-immunoreactive cells. (A) periventricular region of a rat injected with Tat 3 days after treatment. (B) Blood vessels in the corpus callosum of a rat injected once with Tat 7 days after treatment. Arrows point to monocyte-activated microglia labeled with anti-ferritin antibodies.

ratios were generated by averaging the ratios of similarly treated animals. Statistical comparisons between groups for each metabolite were made using a two-way repeated measures ANOVA. Post-hoc analysis was done using Fisher's least significant difference procedure.

RESULTS

Biotinylated Tat was localized to the ependymal cells at 30 min postinjection, and to the periventricular region of rat brains, particularly the corpus callosum and the striatum, at one hour postinjection; however, Tat was not detected beyond 6 hours postinjection (data not shown).

The histopathological features of the rats given a single injection of Tat and rats given repeated injections were very similar. Hence, unless otherwise stated, both sets of rats were referred to as "Tat-injected animals." Enlarged ventricles were noted in Tat-injected animals compared with those injected with BSA (Fig. 1A, B). Although both lateral ventricles were enlarged, the ventricle ipsilateral to the side of injection was larger than the one contralateral to the side of injection. No enlargement was noted on day one, but the lateral ventricles of rats given one Tat injection were enlarged at 3 and 7 days post-Tat injection. Lateral ventricles of animals given repeated Tat injections were subjectively larger than the ventricles of rats after a single Tat injection on the corresponding days. The subarachnoid space was free of inflammation, and no blockage of the cerebral aqueduct or foramen of Monroe was observed in sections examined; however, in view of the choroid plexus swelling (see below) and tissue shrinkage during processing, we can not entirely exclude a functional narrowing of the foramen of Monroe in vivo.

Inflammatory infiltrates were noted in the choroid plexus (Fig. 1D), around subependymal venules (Fig. 1F), and along the ventricular wall (Fig. 1G) in Tat-injected rats, but not in the control animals (Fig. 1C, E). These infiltrates consisted predominantly of neutrophils 1 day after Tat-injection, of mixed macrophage and neutrophil inflammation at 3 days, and of macrophage and lymphocytic infiltration at 7 days. However, the neutrophils remained confined to the lateral ventricle (Fig. 1H) and did not invade the parenchyma. Macrophages and activated microglia were localized mainly in the corpus callosum and in the subependymal region, primarily around the blood vessels as indicated by anti-ferritin staining (Fig. 2). Infiltration of the blood vessel walls and vasculitis were notably absent. Inflammatory infiltrates along the ventricle wall were not immunoreactive with anti-ferritin antisera.

The ependymal lining was covered with neutrophils by day one after a single Tat injection. By day 3, the ependymal cells were absent from the injected lateral ventricle (Fig. 1H). In contrast, the ependymal lining of animals injected with BSA or immunoeluted solution remained intact. Widespread GFAP labeling was present in the ipsilateral and contralateral gray and white matter of Tat-injected animals. GFAP reactivity was most prominent in the corona radiata and corpus callosum, but was also present in the hippocampus and periventricular gray matter, especially around blood vessels. The astrocytes were hypertrophic in these animals (Fig. 3). This was seen at 3 and 7 days after a single Tat injection, but was especially evident in animals injected repeatedly with Tat.

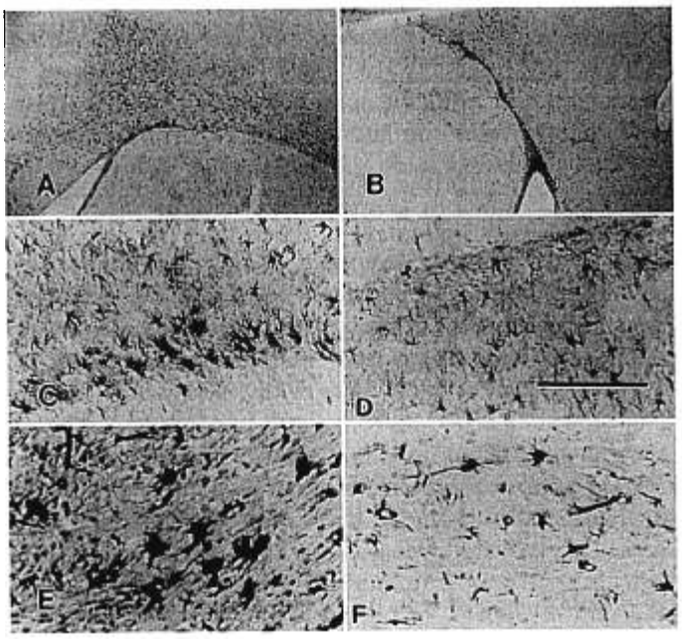

Fig. 3. Tat-induced astrocyte activation. A, C, E: Rats that were repeatedly injected with Tat (same field of view) compared with similarly treated rats injected with BSA in B, D, and F. Astrocytosis was more widespread in the white matter of (A) Tat-injected animals than in (B) BSA-injected animals. (C) Tat-injected rats also showed astrocyte crowding and increased size when compared with (D) BSA-injected controls (D). Astrocyte hypertrophy in (E) Tat-injected animals was more prominent than in (F) BSA-injected animals. Bar represents 500 mm in A and B, 100 mm in C and D, and 50 mm in E and F. A–F: Staining for GFAP.

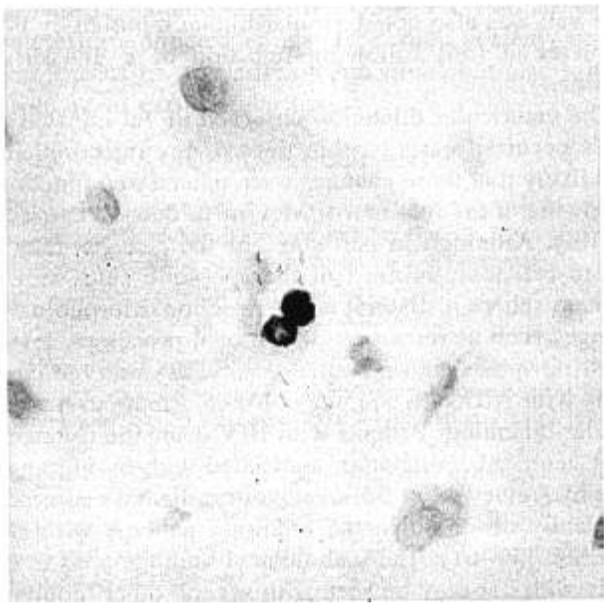

Fig. 4. Tat-induced apoptosis. Two apoptotic cells in the striatum of a rat injected once with Tat. These are probably glial cells based on their nuclear size.

Some GFAP labeling was also noted in the BSA-injected animals, but was much less prominent when compared with Tat-injected animals (Fig. 3).

Tat-injected animals frequently showed cells undergoing cell death as determined by end labeling of DNA fragments (Fig. 4). Results of labeled cell counts from singly and repeatedly injected animals were pooled, as the results were similar in both groups. Although BSA-injected rats showed rare cell death, Tat-injected animals had significantly more cell loss ($p < 0.002$) than the BSA controls: the mean number SEM of apoptotic cells in Tat-treated rats (in the 3 sections examined per animal) was 70.9 ± 27.4 vs 9.4 ± 3.1 for the BSA-treated group. In comparison, the sections examined from one of the rats that was injected with a solution from which Tat had been immunoeluted had 2 apoptotic cells and those from the other rat had 4 apoptotic cells. Apoptotic cells in the Tat-injected animals were generally scattered in the corpus callosum, the periventricular area (including the septal nucleus and the striatum), and within some inflammatory infiltrates.

NMR spectra obtained from brains homogenized in KHCO3-saturated D2O showed a significant ($p < 0.05$) increase in GABA levels in the hippocampus of the Tat injected animals. This was accompanied with a significant difference ($p < 0.05$) in the ratio of the intensity of the resonance of glutamate to that of GABA in the injected hippocampus in the Tat-injected rats when compared with BSA-injected rats (see Table).

DISCUSSION

We and others have shown that Tat is toxic to neurons and, upon action on glial cells in vitro, can induce cytokines. We now demonstrate that even a transient exposure of the brain to Tat can result in profound and progressive neuropathological changes that include an influx of inflammatory cells, gliosis, ventricular enlargement, and cell death. Therefore, it is likely that Tat initiates a cascade of events that self-perpetuates for several days thereafter.

Tat-induced neuropathological changes showed an evolution of inflammatory changes. Early inflammatory changes were noted in the choroid plexus. Inflammation of the choroid plexus has also been observed in a number of retroviral infections, including HIV (42), SIV (43), and Visna (44). In fact, the choroid plexus may be a route of entry for infected cells into the brain (42).

The inflammatory changes in the choroid plexus were followed by periventricular monocytic and lymphocytic infiltrates. Similar perivascular infiltrates have been observed in the brains of patients with HIV infection (5, 45). Mechanisms by which Tat may initiate this inflammatory response include the possibility that Tat itself may act as a chemoattractant molecule for monocytes (46) and

TABLE
Metabolite Levels from Rat Brain Homogenates Determined by NMR Spectroscopy (mean ± SEM)

	Tat-injected	Tat-contralateral	BSA-injected	BSA-contralateral
Hippocampus				
GABA	0.35 ± 0.02*	0.23 ± 0.02	0.19 ± 0.04	0.14 ± 0.02
Glutamate	0.73 ± 0.07	1.25 ± 0.21	1.01 ± 0.13	0.98 ± 0.04
Glutamate: GABA	2.10 ± 0.09*	5.30 ± 0.50	5.60 ± 0.42	7.20 ± 0.92
NAA	0.40 ± 0.01	0.44 ± 0.03	0.37 ± 0.08	0.34 ± 0.02
Cortex				
GABA	0.20 ± 0.17	0.35 ± 0.02	0.37 ± 0.02	0.41 ± 0.06
Glutamate	1.21 ± 0.22	1.04 ± 0.39	0.88 ± 0.06	1.20 ± 0.28
Glutamate: GABA	15.80 ± 11.91	2.95 ± 0.94	2.40 ± 0.22	2.82 ± 0.22
NAA	0.48 ± 0.06	0.44 ± 0.05	0.47 ± 0.01	0.57 ± 0.32
Basal ganglia				
GABA	0.08 ± 0.03	0.07 ± 0.01	0.11 ± 0.02	0.22 ± 0.02
Glutamate	1.0 ± 0.20	1.01 ± 0.02	1.12 ± 0.19	0.81 ± 0.07
Glutamate: GABA	16.86 ± 9.46	14.37 ± 2.43	10.56 ± 1.12	3.65 ± 0.02
NAA	0.35 ± 0.09	0.26 ± 0.02	0.32 ± 0.09	0.38 ± 0.05

GABA = γ aminobutyric acid; NAA = N-acetylaspartatic acid; *$p < 0.05$ compared with BSA injected animals.

that Tat is a potent stimulant for the expression of macrophage chemoattractant protein 1 (MCP-1) in astrocytes (47). Further, MCP-1 levels are increased in the cerebrospinal fluid and brains of AIDS patients with dementia (47). It is thus likely that the release of Tat into the extracellular space may lead to a sequence of events resulting in evolution of the inflammatory response.

Astrocytosis is an early and consistent finding in the brains of HIV-infected patients (48, 49). Hypertrophied astrocytes are seen even in areas without gross histopathological changes (50). Similarly, in the present study, Tat-injected animals also displayed widespread astrocytosis. Tat-treated astrocyte cultures showed increased GFAP production (Conant, personal communication) and the release of cytokines (51), suggesting that Tat may induce astrocyte activation. Further, Tat stimulates the production of tumor necrosis factor-α (TNF-α) in macrophages, an effect that has been implicated in astrocyte proliferation (52). Tat may thus act in an autocrine and paracrine manner to induce astrocytosis.

We have previously shown that Tat may act directly on neurons, causing excitation and neuronal cell death (11, 13, 26), and causing apoptotic changes in a select population of neurons in vitro (53). However, in the present study, since apoptotic cells were observed even 7 days after a single Tat-injection, long after the protein was cleared from the extracellular space, it suggests that other mechanisms causing cytotoxicity may also be involved. These processes may include cytokine-mediated damage. This is consistent with previous observations that Tat derived peptides cause neurotoxicity and cytokine induction in vivo (10, 54). Further, Tat-induced TNF-α production (51) may inhibit glutamate uptake by astrocytes (55) or induce secondary signaling events such as ceramide formation, tyrosine kinase activation, NF-κB activation, calcium mobilization and release, and reactive oxygen species formation, all which have been associated with neurotoxicity (56). The kinetics of these multiple processes may be complex, accounting for the variability of the numbers of dead cells in similarly treated rats. This may also be related to the variable tortuosity of the extracellular space in the brain leading to uneven concentration of Tat, cytokines, glutamate, and other potential toxins. The scattered nature of the apoptotic cells may also suggest a cell-specific vulnerability to Tat. Interestingly, disruption of the ependymal lining of the ventricular wall was also noted, similar to that reported by Phillipion et al (56) following injection of a Tat-derived peptide.

The ventricular dilatation observed in Tat-injected animals occurred early, within days of icv injection. It is thus likely that these changes were related to a functional narrowing of the foramen of Monroe or due to ventricular scarring. Although in part some of the changes may be due to cell loss, as most of the apoptotic cells were in the hemisphere ipsilateral to Tat injection. Morphological changes such as retraction of neuronal processes, loss of dendritic spines, and synaptic density, as observed in patients with AIDS (3, 57), may also contribute to the ventricular dilatation. Patients with HIV dementia frequently have ventricular dilatation associated with basal ganglia atrophy (reviewed in 58). Apoptotic cells have also been frequently observed in the brains of patients with HIV infection (59–61). The pathological findings seen in this study with Tat may be seen with several other biological toxins and in that sense may be considered nonspecific. Nonetheless, similar pathological findings are seen in the brains of HIV-infected patients and we now demonstrate

that an HIV protein, Tat, produces these toxic changes. In contrast, previous studies with another HIV protein, gp120, did not produce similar inflammatory changes or astrocytosis (62), even when similarly injected daily with icv for 14 days (63). Thus, our observations are relevant to the pathogenesis of the illness.

An important observation in the Tat-injected animals was the alteration of the neurotransmitter balance in the hippocampus in the absence of neuronal cell death. This relative increase in GABA indicates a disruption between the excitatory and inhibitory synaptic events. Since these effects were noted 7 days after a single injection of Tat, it suggests that a transient exposure of Tat can cause long lasting functional changes in neurons at distant sites. These observations are important, since it may be possible that at least some of the cognitive impairment in HIV-infected patients may be reversible by the use of appropriate therapeutic intervention.

We propose the following model to explain the wide variety of effects of Tat on the ventral nervous system. Its immediate effects on the brain cells include activation of neurons to produce neuronal firing and changes in intracellular calcium from which the cells may recover or proceed to cell death. Tat also activates glial cells to produce chemokines and cytokines. The further influx of activated mononuclear cells leads to a self-perpetuating cycle of events, which, once initiated, no longer requires the presence of Tat. Although all of the events in this cascade have yet to be identified, it is clear that they eventually lead to cellular loss and/or functional derangement of the central nervous system. The above model suggests several opportunities for intervention in the treatment of patients with HIV dementia. Further, therapeutic efforts directed against Tat should not only be targeted toward its transcription functions, but also toward its role as an extracellular toxin.

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