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Detection of the human immunodeficiency virus regulatory protein *tat* in CNS tissues

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Neuropathologically, human immunodeficiency virus (HIV) is associated with a range of inflammatory disorders, extensive cortical neuronal loss, and dendritic and synaptic damage. Although the mechanisms resulting in these abnormalities are still unclear, the neurotoxic effects are thought to be due in part to viral products including the *tat* gene product. We have previously shown that Tat when presented to neurons extracellularly interacts with neuronal cell membranes to cause neuronal excitation and toxicity in fmole amounts. To determine the role of Tat in mediating HIV encephalitis (HIVE), we detected *tat* mRNA and protein in tissue extracts of nine patients with HIVE and seven patients without HIVE. Despite long autopsy times and significant degradation, *tat* mRNA was detected in 4/9 patients with HIVE but not in any of the seven patients without dementia. Similarly, the *env* mRNA was also detected in 5/9 patients with HIVE but not in the patients without HIVE. However, *vif* mRNA was detected in both groups of patients with (5/9) or without (2/7) HIVE. Using protein extracts from the brains of the same groups of patients we were unable to detect Tat by enzyme linked immunosorbant assay (ELISA) (sensitivity of 2 ng Tat/ml of brain tissue). However, Tat could be detected immunohistochemically and in protein extracts from the brains of rhesus macaques with encephalitis due to a chimeric strain of HIV and simian immunodeficiency virus (SHIV). Our observations support the role of Tat in the neuropathogenesis of HIV and SHIV encephalitis. *Journal of NeuroVirology* (2000) **6,** 145–155.

Keywords: Tat; AIDS; HIV; ELISA; RT–PCR; Southern blot

Introduction

The human immunodeficiency virus (HIV), the etiological agent of the acquired immunodeficiency syndrome (AIDS) can infect the central nervous system (CNS), leading clinically to several neurologic disorders. HIV-associated dementia is one of the most common and devastating of these neuropsychiatric conditions (Price *et al*, 1988; Achim *et al*, 1991; Asare *et al*, 1996). Neuropathologically HIV can cause a spectrum of damage, including inflammatory disorders such as HIV encephalitis and leukeoencephalopathy (Budka *et al*, 1991); significant and regionally variable cortical neuronal loss (Wiley *et al*, 1991; Asare *et al*, 1996; Everall *et al*, 1991, 1993) and dendritic and synaptic damage (Masliah *et al*, 1992, 1996). Viral proteins have been detected in microglial cells, macrophages, multinucleated giant cells and other inflammatory cells, and these have been suggested to act as the principal cellular viral reservoir in the brain (Gendelman *et al*, 1997). As direct neuronal infection by HIV has only rarely been shown (Nuovo *et al*, 1994; Bagasra *et al*, 1996), it follows that neuronal damage would require neurotoxic agents to be produced by other cells within the brain. Two potential neurotoxic candidates are viral proteins and excess cytokines. Although several HIV proteins have been shown to have neurotoxic properties, the envelope glycoprotein gp120 and the regulatory protein Tat, have been studied in much detail (Nath and Geiger, 1998).

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Gp120, or its fragments, have been shown to be neurotoxic, and stimulate a large increase in intracellular Ca^{2+} in cultured rat hippocampal neurones (Brenneman *et al*, 1988; Dreyer *et al*, 1990), and in synaptosomes (Nath *et al*, 1995). Gp120 can also induce free radical generation (Foga *et al*, 1997) and cytokine production, such as tumour necrosis factor-alpha (TNF-α), which has also been observed to be toxic and induce apoptosis (Lipton, 1992b). The gp120-induced Ca^{2+} influx, probably via voltage-gated and glutamate linked Ca^{2+} channels, can be blocked by Ca^{2+} channel and N-Methyl-D-Aspartate (NMDA) receptor antagonists (Lei *et al*, 1992; Lipton, 1991, 1992a; Muller *et al*, 1992; Tak-Man *et al*, 1992). The consequences of glutamate receptor overstimulation is likely to be detrimental and is implicated in HIV associated excitotoxic damage (Lipton, 1992b). However, gp120 has not yet been demonstrated in the brain tissue of individuals who died of AIDS or in the gp120 transgenic mouse model, which produces similar CNS damage to that found in AIDS (Toggas *et al*, 1994).

HIV-1 tat is a non-structural viral protein, secreted extracellularly by infected cells (Frankel and Pabo, 1988; Ensoli *et al*, 1990, 1993; Chang *et al*, 1997), which is a potent stimulator of HIV-1 transcription and therefore viral replication (Desai *et al*, 1991; Drysdale and Pavlakis, 1991). Several studies have shown that the Tat protein may alter the blood brain barrier permeability, and affect the expression of a number of cellular regulatory factors and cytokines (De la Monte *et al*, 1988; Nath and Geiger, 1998). In fact Tat is a potent stimulant for TNF-α in macrophages (Chen *et al*, 1997) and monocyte chemoattractant protein in astrocytes (Conant *et al*, 1998). In-vitro *tat* transfected cells undergo apoptosis (Benjouad *et al*, 1993; Purvis *et al*, 1995), and Tat induced neurotoxicity has been demonstrated on cultured human foetal brain cells, rat hippocampal cells and neural cell lines (Sabatier *et al*, 1991; Magnuson *et al*, 1995; Strijbos *et al*, 1995; Weeks *et al*, 1995; Nath *et al*, 1996; New *et al*, 1997). *Tat* when presented in fmole dosage can interact directly with neuronal cell membranes to cause neuronal excitation (Cheng *et al*, 1998). Cytotoxicity was significantly inhibited by blockade of excitatory amino acid receptors (Magnuson *et al*, 1995). Therefore suggesting, like gp120, excessive excitation of glutamate receptors could underlie Tat-neurotoxicity. The extracellular presence of Tat is well established. Tat is essential for viral replication and is formed from the viral genome in infected cells, and is released from infected lymphoid (Frankel and Pabo, 1988; Ensoli *et al*, 1993) and glial cells (Tardieu *et al*, 1992). Tat is released from HIV-1 infected cells by a leaderless secretory pathway, in the absence of cell death at the moment of highest gene expression (Chang *et al*, 1997). Tat can be detected in the sera of HIV infected individuals (Westendorp *et al*, 1995), and infected glial cells produce much larger amounts of *tat* transcripts as compared to that of p24 or gp41 (Tornatore *et al*, 1994). Nonetheless, detection of Tat protein by immunohistochemistry has been difficult due to cross reactivity of the Tat antisera with normal brain proteins (Parmentier *et al*, 1994). In this study, we have developed and utilised sensitive and specific assays to detect the presence of *tat* transcripts and protein in the frontal cortex from the brains of individuals who died of AIDS. We also demonstrate the presence of Tat protein in macaques with encephalitis due to a chimeric strain of HIV and Simian immunodeficiency virus (SHIV).

The immunopathological and neuropathological manifestations of SHIV infected animals most closely resemble HIV infection in humans. The virus is dual tropic for macrophage lineage cells and CD4 cells. It invades the brain early in the course of infection and causes a productive replication in brain cells. The use of this animal model provides the advantage of obtaining brain samples with short autopsy times, hence we also examined brain tissue from SHIV infected animals for the presence of Tat.

Results

Tat protein estimation

The clinical neuropathological examination revealed that none of the control cases showed detectable abnormalities. While in the HIV group nine had HIV encephalitis; one had minimal pathology, such as astrocytosis and monocytic perivascular cuffing; one had an opportunistic infection, cytomegalovirus encephalitis; and five had no detectable abnormalities. These findings are summarised in Table 1. The mean age of the control group was 43 ± 21 years, and that of the HIV group 37 ± 11 years. The difference between these mean ages was not statistically different (P=0.7). The mean post mortem delay between the groups was 32 ± 16 and 64 ± 41 h for controls and HIV respectively, this was also not statistically significant (P=0.11).

The ELISA was standardised against varying concentrations of recombinant HIV-1 Tat, in lysing buffer at varying pH's, in the presence and absence of brain homogenate. Replicable standard curves were obtained for each experiment. From this the sensitivity of the assay was estimated to have a lower threshold of detection of Tat at 2 ng/ml (20 nM) (Figure 1). The total mean protein concentration in μg/ml for the samples of the two groups, as estimated by optical density readings at 280 nm (Genesys), were found to be 66 ± 22 μg/ml in the control group and 22 ± 12 μg/ml for the HIV group, a difference of 66%, which was statistically significant (P=0.0001).

Normally 10–12% of wet brain weight should be proteins (Pitlick and Nemerson, 1976). Our sample supernatant was diluted to a concentration of 1 mg/

ml (wet weight of grey matter) in lysing buffer. Therefore, the total amount of protein was expected to be in the range of 100–120 μg/ml. We were able to detect approximately 66% of the total protein in the controls. The amount of protein in the HIV brain samples was only one third of that in the controls. The loss of protein suggests that degradation may have taken place at post mortem, or more likely that the protein was lost in the membrane fractions during dilution. The stability over time of the rHIV-1 Tat protein in brain tissue was also assessed. It was found that in frontal cortical tissue homogenate, incubation of 25 ng/ml of the recombinant protein with brain homogenate over a period of time ranging from 15–360 min at 37°C did not result in loss of detection. In addition, storing the brain homogenate with rHIV-1 Tat for a period of 3 days at −70°C did not affect the assay which was still able to detect the rHIV-1 Tat.

Table 1 Summary of pathological and molecular findings in ten controls and 16 individuals who had died of AIDS.

Case	*Sample*	*Sex*	*Age (Years)*	*PM delay (H)*	*Neuro Pathology*	*vif*	*env*	*tat*
C1	51	M	48	28	Normal			
C2	8	M	48	59	Normal			
C3	2	M	64	48	Normal			
C4	28	M	22	45	Normal			
C5	44	M	21	37	Normal			
C6	29	F	20	38	Normal			
C7	49	M	16	14	Normal			
C8	5	M	63	26	Normal			
C9	31	M	72	12	Normal			
C10	4	M	51	15	Normal			
		Average	42.5	32.2				
		S.D.	19.9	15.1				
H11	21	M	28	48	HIVE	+	+	
H12	27	M	29	24	HIVE	+	+	+
H13	20	F	27	96	HIVE			
H14	18	M	39	24	HIVE			
H15	43	M	32	72	HIVE	+	+	+
H16	1	M	40	80	MinPath			
H17	19	M	31	20	NDA			
H18	3	M	41	120	NDA	+		
H19	35	M	25	96	NDA			
H20	47	M	31	120	HIVE	+	+	+
H21	10	M	66	69	HIVE			
H22	38	M	37	45	NDA			
H23	9	M	56	10	CMV			
H24	23	M	41		HIVE	+	+	+
H25	7	M	31	120	NDA	+		
H26	40	M	40	10	HIVE			
		Average	37.1	63.6				
		S.D.	10.9	40.9				

NDA, no detectable abnormalities; HIVE, HIV encephalitis; CMV, cytomegalovirus; MinPath, minimal pathology.

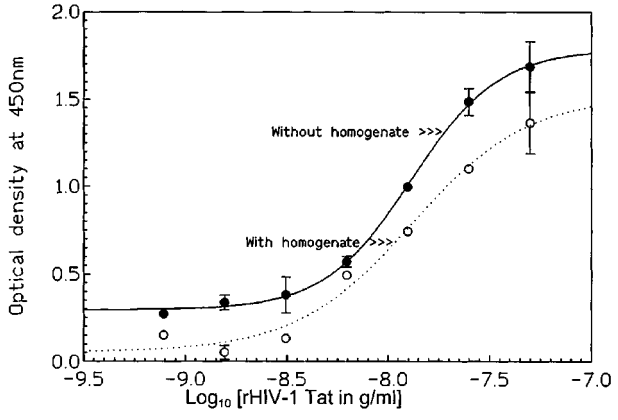

Figure 1 Standard curve for the recombinant HIV-1 *tat* (1-86) ELISA assay demonstrating the detection in the presence and absence of control human brain homogenate (1 mg/ml) in lysing buffer. Error bars are the standard error of mean (s.e.m.). The brain homogenate was prepared and the ELISA assay conducted as described in Materials and methods. The human brain homogenate used was sample C4(#28).

Examination of the ten controls and 16 HIV cases did not reveal any detectable levels of HIV-1 Tat. The $OD_{450\ nm}$ readings obtained, gave values for HIV-1 Tat below the assay's threshold as determined from the standard curve (Figure 1). These were a mean $OD_{450\ nm}$ of 0.076 for the control group, and 0.118 for the AIDS group. Thus all the samples were deemed negative for the presence of HIV-1 Tat.

tat *mRNA Detection*

Integrity of RNA in autopsy specimens was assessed initially. Total RNA isolated from each specimen was analysed by electrophoresis. Three samples showed the presence of both the 28S and 18S rRNA, another six samples showed only 18S rRNA. In all other samples only degraded RNA was detected (Figure 2A). In control samples prepared from fresh mouse brain, clear 28S and 18S rRNA bands were noted (Figure 2B). Nonetheless, β Actin mRNA could be amplified from all samples (Figure 2C) demonstrating relative preservation of mRNAs compared to rRNA in these autopsy samples.

To determine the sensitivity of mRNA detection, various concentrations of cDNA sample from 8E5/LAV cells mixed with 0.1 μg of known HIV negative cDNA were probed by Southern blot analysis. The sensitivity of detection for *env* mRNA was 16 cells, while that of the *tat* mRNA was 1.6 cells as shown in Figure 3. The sensitivity for *vif* detection was 1.6 cells (data not shown). Detection of *tat*, *vif* and *env* mRNA in autopsy specimens revealed that in seven of 16 HIV group samples (H11(#21), H12(#27), H15(#43), H18(#3), H20(#47), H24(#23), H25(#7)) *vif* mRNA was detected (Figure 4A). In four of these samples (H12(#27), H15(#43), H20(#47), H24(#23)), *tat* mRNA could be detected (Figure 4B) and in five samples (H11(#21), H12(#27), H15(#43), H20(#47), H24(#23)) *env* mRNA was present (Figure 4C). HIVE was present in all cases in which *tat* and *env* mRNA was detected, and in all but two cases in which *vif* mRNA was present. HIV gene products were not detected in any of the control samples.

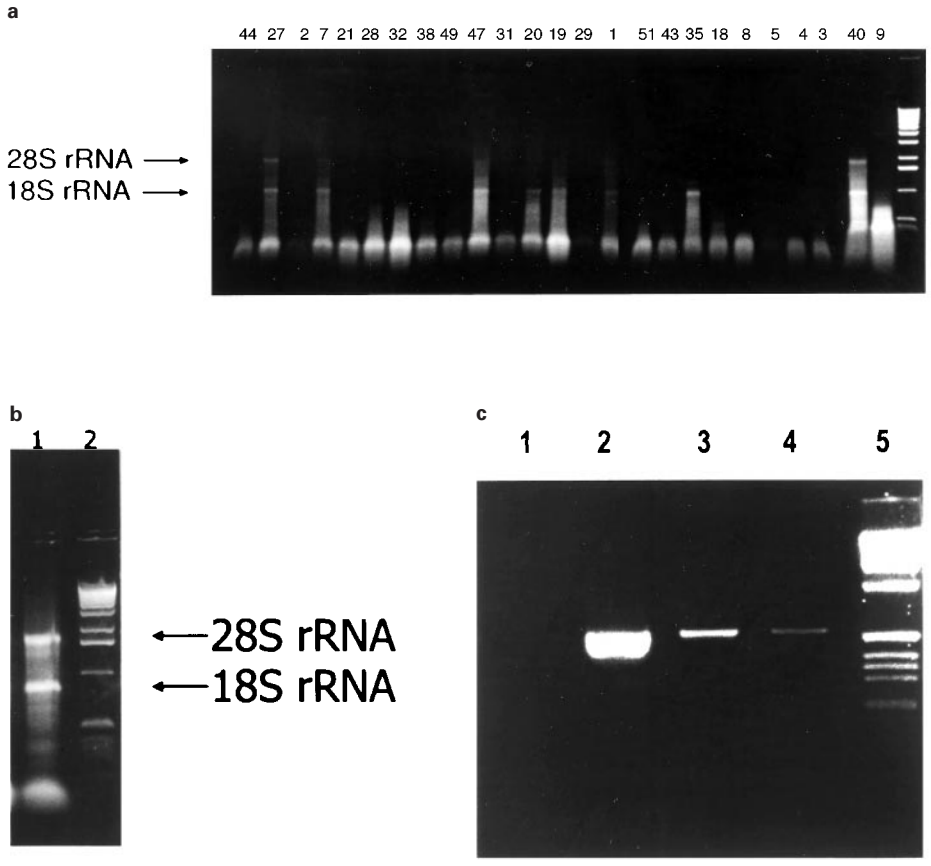

Figure 2 (**A**) Electrophoresis of total RNA on agarose gel. Numbers above each lane refer to the original sample number code, which was assigned prior to the experiment to ensure that the investigator was blind to the sample diagnosis. i.e. control or HIV specimen. The sample number list is presented in Table 1. Specimen H12(#27), H25(#7) and H26(#40) show the presence of 28 S and 18 S rRNA bands, specimen H13(#20), H17(#19), H16(#1), H19(#35), H20(#47), H23 show only 18 S rRNA bands the remaining samples show the presence of degraded RNA. (**B**) Mouse brain RNA similarly analysed by agarose gel electrophoresis shows prominent 28 S and 18 S rRNA bands in lane 1 together with the molecular marker in lane 2. (**C**) Detection of β-actin mRNA in brain samples of patients with HIV infection: mRNA for β-actin was detected in brain extracts by RT–PCR using previously published primer sequences (Chen *et al*, 1997) and analysed by agarose gel electrophoresis. Lane 1 represents a control sample without the template. Lane 2 represents a positive control (normal mouse brain). Representative samples are shown in lanes 3 and 4, samples H11(#21) and C4(#28) respectively. Molecular weight markers are shown in the last lane (lane 5).

Tat detection in SHIV infected animals

Immunohistochemistry showed focal areas of Tat positive mononuclear cells. Cytoplasmic staining was noted in cells in the white matter and in the adjacent cortex in close vicinity to neurons. Tat immune reactivity was also noted in the matrix surrounding some of the Tat positive cells, suggesting an extracellular release of Tat from these cells. No staining was noted in the sections from an uninfected macaque (Figure 5). Clearly detectable bands of Tat protein were seen in brains of animals 16B and 23A by Western blot analysis. Both of these animals had lentiviral encephalitis (Raghavan *et al*, 1997). Faint bands were present in the brain and lung tissues of animal 23E. Tat could not be detected in the lungs of animals 16B and 23A (Figure 6).

Discussion

In this study we have probed for the presence of both Tat protein and mRNA in HIV infected human brain tissue and Tat protein in SHIV tissues. Using recombinant Tat, we have developed a specific assay that was able to detect Tat protein, in either lysing buffer or in lysing buffer containing brain homogenate, with a sensitivity threshold of 2 ng/ml. This is equivalent to 138 pM for a Tat protein of 86 amino acids with a molecular weight of 14.4 kD. In testing the frontal cortical brain samples from both the control and HIV infected groups, our assay did not detect any appreciable amounts of Tat protein. However our assay may not have been sensitive enough since 20–200 fmol of Tat may be sufficient to cause neuronal dysfunction (Cheng *et al*, 1998). Further even though our observations may suggest

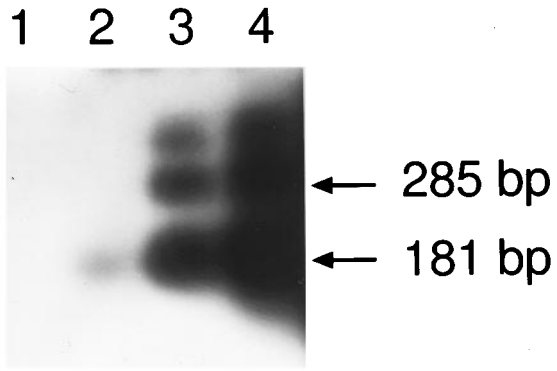

Figure 3 Sensitivity measurement. Serial dilutions of cDNAs from 8E5/LAV cells, lane 1: 0 cells; lane 2: 1.6 cells; lane 3: 1.6×10^1 cells; lane 4: 1.6×10^2 cells were used in PCR in the presence of about 0.1 μg of HIV negative cDNA and electrophoresed in a 2% agarose gel and transferred to a nitrocellulose filter. The blot was hybridised with ^{32}P-labelled *tat* and *rev* probes. The autoradiogram was exposed for 18 h at -70°C. The positions of the *tat* cDNA (181 bp) and of the *env* cDNA (285 bp) are indicated. The sensitivity of detection for *tat* was 1.6 infected cells and *env* was 16 infected cells.

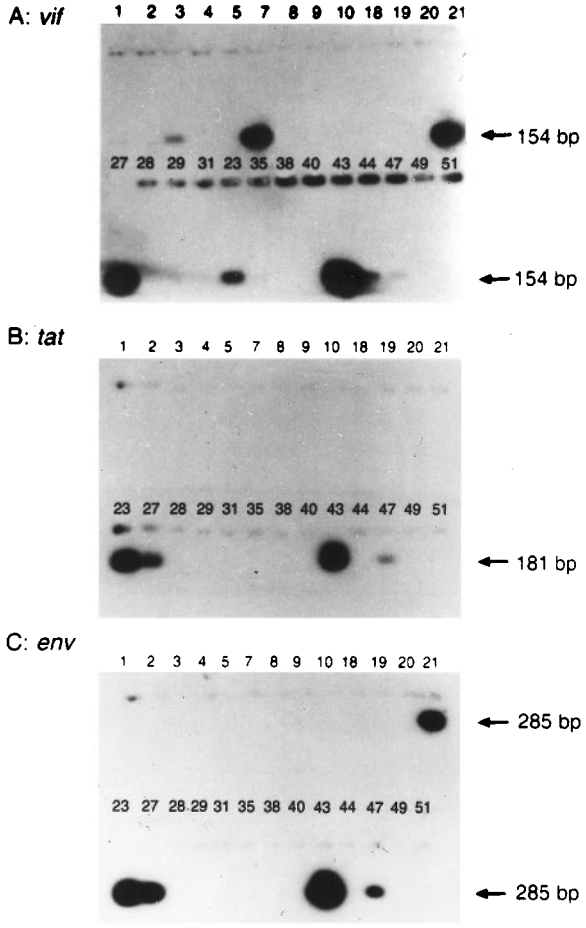

Figure 4 Detection of HIV *vif*, *tat* and *env* mRNAs in the brain specimens. cDNA derived from RNA of brain samples was used in PCR with specific primers for *vif* and primers that allow amplification of only spliced mRNAs of *tat* and *env*. PCR products were electrophoresed in a 2% agarose gel and transferred to nitrocellulose filters. The blots were hybridised with ^{32}P-labelled *vif*, *tat* and *env* probes. The autoradiogram was exposed for 18 h at -70°C. Lane numbers equivalent to the specimen numbers. (**A**) displays amplified products of *vif*, specimens H11(#21), H12(#27), H15(#43), H18(#3), H20(#47), H24(#23), H25(#7) show positive hybridisation signals. (**B**) displays amplified products of *tat*, specimens H12(#27), H15(#43), H20(#47), H24(#23) show positive hybridisation signals. (**C**) displays amplified products of *env*, specimens H11(#21), H12(#27), H15(#43), H20(#47), H24(#23) show positive hybridisation signals.

that Tat protein may be stable over time and that freezing and thawing the brain homogenate sample did not affect protein detection, the possibility that the amount of protein degradation in the autopsy sample that may have occurred immediately after *post mortem* needs to be considered since the proteases would be most active at that time. This is particularly important for proteins present in the extracellular compartment. This is supported by our ability to detect Tat easily in the SHIV infected animals with encephalitis where the tissue was snap frozen with necropsy of <1 h. Despite the prolonged autopsy times and significant degradation of RNA in the human brain samples we were able to detect mRNA for β actin and HIV genes by PCR amplification techniques. *vif* mRNA was detectable in almost 50% of the group, the majority of which had HIVE. However, *env* and *tat* mRNA was detected only in cases with HIVE. These observations are consistent with those of other investigators who have also found increased transcripts for *tat* in patients with HIV dementia (Wesselingh *et al*, 1997; Wiley *et al*, 1996). We clearly demonstrate Tat positive cells in macaques with lentiviral encephalitis. An interesting observation is that the extracellular matrix surrounding some of these Tat-positive cells also showed Tat immunoreactivity. These observations suggest that Tat was likely released from these cells. In fact, several *in vitro* studies have shown that HIV infected cells may release Tat extracellularly (Ensoli *et al*, 1993; Tardieu *et al*, 1992) by active secretion via a leaderless pathway (Chang *et al*, 1997). We also demonstrate Tat positive cells in close vicinity of neurons. Tat released from these cells would have the opportunity to interact with other infected and uninfected cells. We and others have previously shown that Tat causes neuronal excitation followed by cell death (Magnuson *et al*, 1995; Nath *et al*, 1996; Cheng *et al*, 1998) while it activates glial cells to produce cytokines and chemokines (Chen *et al*, 1997; Conant *et al*, 1998).

Several other factors need to be considered while measuring concentrations of Tat in brain tissue (Nath *et al*, 1998). Once it is present in the extracellular space, it may either interact with cell

Figure 5 Immunohistochemical detection of Tat in brain. (**A**) A focal region in the cell (arrow) shows intense staining for Tat. Tat staining is also seen in the adjacent matrix. (**B**) Tat staining is noted extracellularly in a configuration suggesting release from the cell in the center. (**C**) Several Tat positive cells are seen (arrows). (**D**) perivascular cells and matrix staining for Tat. (**E**) absence of Tat staining is seen in the uninfected brain tissue.

membranes of neurons and glial cells to cause neurotoxicity (reviewed in Sykova, 1997) or it may be taken up by neighbouring cells and transported to the nucleus (Frankel and Pabo, 1988; Mann and Frankel, 1991; Ensoli *et al*, 1993; Ma and Nath, 1997). Cellular uptake of Tat is determined by the N-terminal region of Tat formed by the second exon (Ma and Nath, 1997). In a recent study, we determined that this region of *tat* has a large number of mutations in patients with HIV dementia (Fawell *et al*, 1994; Mayne *et al*, 1998) suggesting that the cellular uptake of Tat in these individuals may be impaired leading to increased extracellular levels. In the present study, while preparing protein extracts, we discarded the nuclei, hence only extracellular, cytoplasmic, or membrane-bound Tat was measured. The extracellular space of the brain comprises of only 20% of the total brain volume and factors such as glial swelling, hypertrophy (gliosis), changes in pH, potassium or sodium concentrations can cause significant shrinking of the extracellular space by even 50%. The extracellular space is also not homogenous but varies in different regions. For example it is much more compact in the hippocampus compared to the cortex and within the hippocampus it is much more compact within the CA1 region as compared to the CA3 region. Further, once Tat is available in the extracellular space it's ability to diffuse through the extracellular space would be impacted by the tortuosity of the space and the presence of large molecules such as glycoaminoglycans and glycoproteins that are present in the extracellular space but not in the CSF. Additionally, the size and charge of Tat itself would influence its migration in the extracellular space. One might thus expect that the concentrations of Tat would be variable in the brain reaching very high concentrations in some regions and absence of the molecule in other regions. Our observation that focal areas of Tat positive cells were seen by immunohistochemistry supports this concept. As shown for well accepted neurotoxins glutamate and kainate, alterations in the size of the extracellular space may change what was a physiological concentration of glutamate to a pathological concentration (Westendorp *et al*, 1995).

It has been shown that even a transient exposure of the brain to Tat can result in profound and progressive neuropathological changes that include influx of inflammatory cells, gliosis, ventricular enlargement and cell death (Wiley *et al*, 1991). It is likely that Tat initiates a cascade of events that self perpetuates for several days thereafter. Similarly *in vitro* experiments show that neuronal excitation occurs within milliseconds of Tat exposure (Jones *et al*, 1998; Magnuson *et al*, 1995) which are followed by increases in intracellular calcium a few minutes later. Further, exposure of the glial cells in culture to Tat for a few minutes leads to cytokine production several hours later (Wiley *et al*, 1991). Thus the continued presence of Tat may not be necessary for implicating it as one of the factors causing neural dysfunction. Our observations support the role of HIV-1 Tat protein in the pathogenesis of HIV encephalopathy.

Materials and methods

Brain specimens

Brain tissue from the frontal cortex of ten controls and 16 individuals who had died of AIDS was obtained from the AIDS and Neurodegenerative Brain Bank, London, England. Clinical details were provided from scrutiny of the medical records. The control group (nine males and one female) had died of a variety of systemic illnesses and had an age range of 16 to 72 years. The HIV group (15 males and one female) were all clinically diagnosed as having at least one of the AIDS defining illnesses. The age

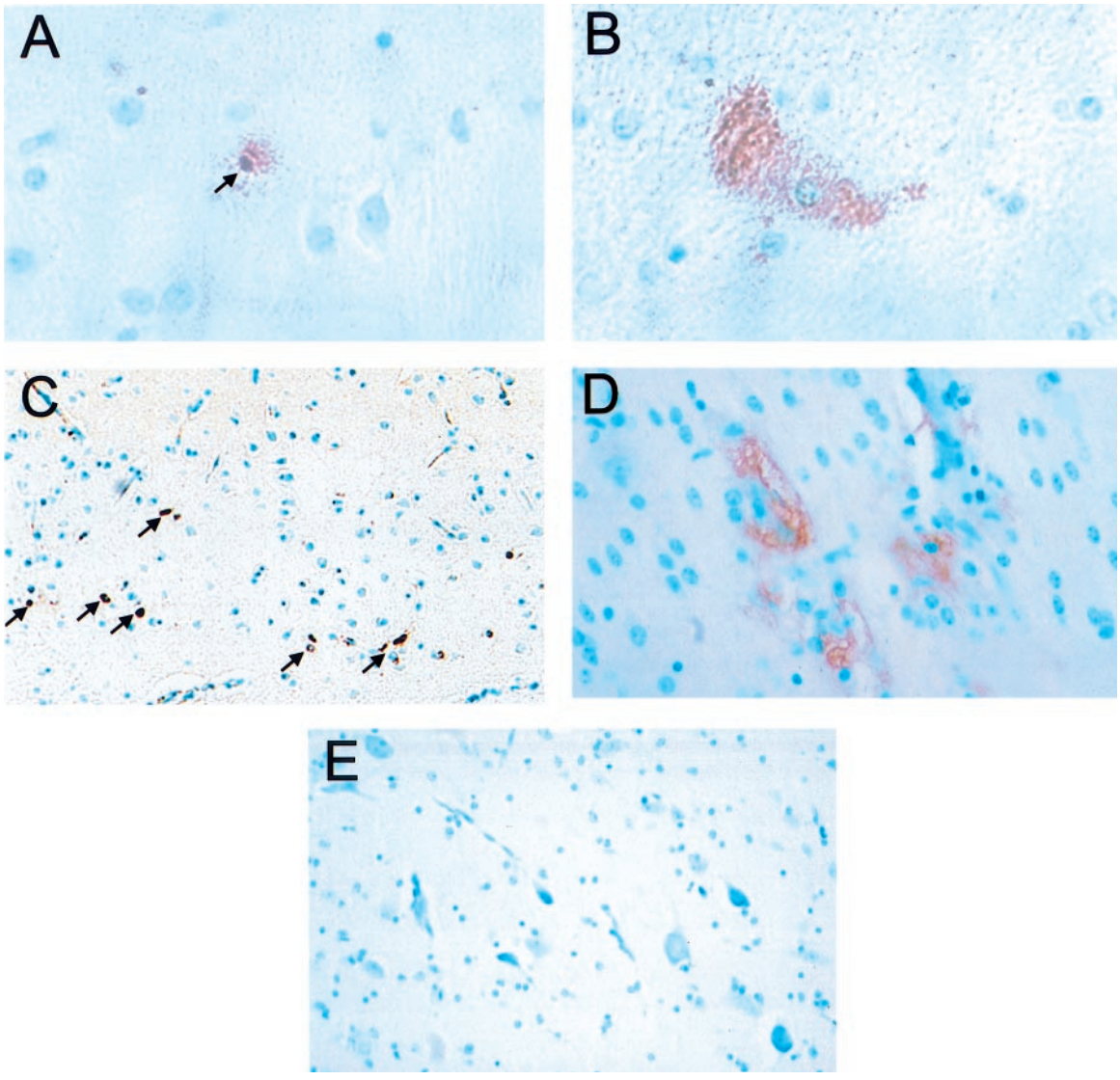

Figure 6 Brain and lung homogenates were prepared from three rhesus macaques infected with $SHIV_{ku2}$. Protein extracts were analysed by 10% SDS–PAGE followed by Western blot analysis. Thirty μg of protein were loaded on to each of lanes 3 to 8. Lane 1 represents 10 μg $Tat_{1\text{-}72}$; Lane 2 represents 1 μg $Tat_{1\text{-}72}$; Lanes 3 and 4 represents brain and lung samples respectively from animal 16B. Lanes 5 and 6 represent brain and lung samples respectively from animal 23E. Distinct bands for *Tat* are seen in lanes 3 and 5; both of these animals had lentiviral encephalitis. The molecular mass of *Tat* from each of the animals is slightly higher than that of the $Tat_{1\text{-}72}$ (lanes 1 and 2) indicating that it represents full length *Tat*.

range was 25 to 66 years. Clinical neuropathological examination was performed in each case (Table 1).

Grey matter from the frontal cortex of fresh frozen brain tissue was carefully dissected from the white matter and weighed on ice. The cortical gray matter samples were then sonified on ice in a known volume of lysing buffer (150 mM NaCl, 50 mM Tris-HCl, 0.1% sodium dodecyl sulphate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 200 μg/ml phenyl-methyl-sulphonyl-fluoride, 1 mM ethylene-diamine-tetra-acetic-acid (EDTA) and 0.02% sodium azide; pH 8.0). They were then spun at 13 000 r.p.m. for 10 min and the pellet discarded and supernatant collected and stored at -70°C. These samples were used for the enzyme-linked immunosorbent assay (ELISA).

Brain and lung tissues from three rhesus macaques (designated 16B, 23A and 23E) infected with a neurovirulent chimeric simian/human immunodeficiency virus ($SHIV_{KU\text{-}2}$) were obtained. This viral strain has the Tat gene of HIV-1 HXB2. Mode of infection and neuropathological findings of these animals have been published previously (Raghavan *et al*, 1997). The animals were perfused with formal saline and the left hemisphere of 10% formalin-fixed brain was dissected and snap frozen over dry ice within an hour of necropsy. Animal 23E had

latent SHIV infection of the CNS but did not have any neuropathological abnormalities or opportunistic infections. Animals 16B and 23A had multinucleated giant cell encephalitis (Raghavan *et al*, 1997). An uninfected animal (NRS) was used as a control.

Enzyme-linked immunosorbent assay
For the detection of Tat, 96 well microtiter plates (Nunc, Maxisorb) were coated overnight at 4°C in a humid chamber with 5 μg/ml goat anti-mouse IgM (Crawley) diluted in PBS (pH 7.4). Non-specific binding was blocked by 5 mg/ml bovine serum albumin (Sigma) in PBS. The plates were then washed with PBS containing 0.2% Tween-20 followed by incubation with 2 μg/ml mouse anti-Tat IgM (MRC AIDS Reagent Project, EVA3069.2) in blocking solution containing 0.1% Tween-20. Washed then incubated with sample supernatant diluted to a concentration of 1 mg/ml (wet weight of grey matter) in lysing buffer. The plates were washed again and incubated with 2 μg/ml mouse anti-Tat IgG (MRC AIDS Reagent Project, ARP352) in blocking solution/0.1% Tween-20. They were washed and finally incubated with a sheep anti-IgG-heavy chain specific horseradish peroxidase conjugated antibody (Serotec) diluted 1 : 1000 in PBS. The ELISA reaction was developed with a 97 mM sodium acetate/3 mM citric acid buffer (pH 6.0) containing 100 μg/ml 3,3′,5,5′-Tetra-methyl-benzidine (Sigma) and 0.03% H_2O_2. The reaction was stopped with 10% sulphuric acid and the plates read at 450 nm. The Tat protein concentration was determined from a standard curve containing recombinant HIV-1 Tat (rHIV-1 Tat) (MRC AIDS Reagent Project, EVA658) diluted in lysing buffer containing 1 mg/ml control brain tissue homogenate. The total amount of tissue protein present in the samples was estimated by an optical density reading at absorbance 280 nm where 1.0 $OD_{280\ nm}$ is equated to 1 mg/ml protein. All analyses were performed blind to the diagnostic group of the tissue sample. The statistical analysis was performed by Mann-Whitney *U*-test (Instat, UK), and the standard curves were calculated on Graphplot.

Reverse transcriptase-polymerase chain reaction

Cell line 8E5/LAV, a cellular clone isolated from a chronically HIV-infected T-cell line (Folks *et al*, 1986; Ramazzotti *et al*, 1996), has a single integrated provirus that is constitutively expressed. These cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, and used as a control for HIV mRNA detection as well as for determining the sensitivity of detection assay. All analyses were done by investigators blinded to the HIV status of the patients. 8E5/LAV was obtained from the NIH AIDS Research and Reference Reagent program.

RNA preparation and RT–PCR analysis About 100 mg of frozen brain tissue from each specimen was homogenized in TRIZOL reagent (Gibco BRL). Total RNA was isolated as per manufacturer's protocol from each specimen or 8E5/LAV cells. Three μg of total RNA from each sample were reverse transcribed into cDNAs in a volume of 15 μl using First-Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR was performed using 0.5 μl of cDNA solution (representing about 0.1 μg of cDNA). PCR mixture was constituted with PCR buffer (10 mM Tris-HCL, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% (w/v) gelatin), 0.2 mM dNTPs, 0.25 μM of each 5′ and 3′ primers and 2.5 units of *Taq* polymerase in a total volume of 50 μl. PCR reaction was carried out in a DNA thermal cycler (PTC-100 MJ Research Inc.) for 3 min at 95°C followed by 30 cycles of denaturation at 95°C for 35 s, annealing at 65°C for 1 min and extension at 75°C for 2 min. The oligonucleotide sequences for the primers and probes used are given in Table 2. Both *tat* and *env* amplification utilised the same sense primer that binds to a region of the 5′ end of LTR mRNA. Antisense primers are complementary to specific exon sequences of *tat* and *env* (Dawood *et al*, 1992). *Vif* primers were designed from conserved regions of the gene (Table 2). Amplification for β actin was also performed in each sample, using previously published primers (Chen *et al*, 1997; Munis *et al*, 1992).

Table 2 Oligonucleotides used for amplification and detection of *env*, *tat* and *vif* mRNAs.

MRNA species	*Primer and probes sequences*	*Size of PCR product*
Env	(sense primer) 5′-ACGGCAAGAGGCGAGGGGAGGCGACTG-3′ (antisense primer) 5′-CTTCACTCTCATTGCCACTGTCTTCTGC-3′ (probe) 5′-CGGAGACAGCGACGAAGACCTCCTCAAGGC-3′	285 bp
Tat	(sense primer) same as the one for *env* (antisense primer) 5′-GCAATGAAAGCAACACTTTTTACAATA-3′ (probe) 5′-AGAGCCCTGGAAGCATCCAGGAAGTCAGC-3′	181 bp
Vif	(sense primer) 5′-ATTGTGTGGCAAGTAGACAGGATGA-3′ (antisense primer) 5′-CTAGTGGGATGTGTACTTCTGAACT-3′ (probe) 5′-AGTTTAGTAAAACCACATATGTATGTTTCA-3′	154 bp

Southern blot hybridisation Fifteen μl of PCR product were electrophoresed through a 2% agarose gel and transferred to a nitrocellulose membrane (NEN products). Blots were prehybridized in $5\times$ SSPE ($1\times$ SSPE=0.15 M NaCl; 0.01 M sodium phosphate monobasic; 0.001 M EDTA), 1% SDS and $10\times$ Denhardts' solution ($1\times$ Denhardts' solution=0.02% polyvinylpyrrolidone; 0.02% ficoll 400; 0.02% bovine serum albumin) at 61°C for 1 h. Internal oligonucleotide probes labelled with ^{32}P-ATP by T4 polynucleotide kinase were added to the prehybridization buffer with 10^6 d.p.m./ml and hybridised at 61°C for 12 h. Blots were washed at room temperature in $2\times$SSC ($1\times$SSC=0.15 M sodium chloride; 0.015 M sodium citrate) and then twice at 61°C in $2\times$SSC/1% SDS, finally, washed twice at room temperature in $0.1\times$SSC. The blots were developed by autoradiography.

Sensitivity measurement The RNA pellet isolated from 4.9×10^6 8E5/LAV cells was redissolved in 8 μl of DEPC-treated water and used to synthesise cDNA in a total volume of 15 μl. 0.5 μl of tenfold serial dilutions of the cell cDNA ranging from 1.6×10^{-1} to 1.6×10^4 cells was added to the PCR reaction mixture as described above and mixed with 0.5 μl of known HIV negative brain cDNA.

Western blot analysis One gram of brain or lung tissue from each of the animals was homogenised in 400 μl of lysing buffer on ice. All samples were clarified by centrifuging at 2000 r.p.m. for 10 min, protein concentrations were determined by a colorimetric Biorad assay, aliquoted and stored at -70°C till further analysis. Each sample was resolved by a 10% SDS–PAGE, transferred to a nitrocellulose membrane and analysed by immunoblotting using rabbit antisera to Tat that had been preabsorbed on protein extracts from normal human brain tissue, and a chemiluminescent detection technique (Pharmacia). The sensitivity of detection for Tat by this technique was 100 pg. Recombinant $Tat_{1\text{-}72}$ was used as a positive control. Antisera to Tat and recombinant Tat protein were prepared as described previously (Ma and Nath, 1997).

Immunohistochemistry Paraffin embedded formalin-fixed sections from the hippocampus and thalamus of each of the rhesus macaques were reacted over night with rabbit polyclonal antisera to Tat (1 : 50 dilution) followed by incubation with anti-rabbit antisera conjugated to horseradish peroxidase for 90 min. Signal was amplified using the TSA-indirect method (Tyramide signal amplification; NEN Life Sciences) following manufacturer's instructions: Briefly, sections were incubated with 1 : 50 dilution of the biotinylated tyramide for 10 min followed by incubation with streptavidin conjugated to horseradish peroxidase for 30 min. Diaminobenzidine was used as a chromogen. Sections were counterstained with methyl green, dehydrated in graded alcohols, and mounted with Permount (Fisher).

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