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## Detection of Anti-Tat Antibodies in CSF of Individuals with HIV-Associated Neurocognitive Disorders

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### Abstract

Despite major advances in the development of antiretroviral therapies, currently available treatments have no effect on the production of HIV-Tat protein once the proviral DNA is formed. Tat is a highly neurotoxic and neuroinflammatory protein, but its effects may be modulated by antibody responses against it. We developed an indirect enzyme linked immunosorbent assay and measured anti-Tat antibody titers in CSF of a well characterized cohort of 52 HIV-infected and 13 control individuals. We successfully measured anti-Tat antibodies in CSF of HIV infected individuals with excellent sensitivity and specificity, spanning a broad range of detection from 10,000 to over 100,000 relative light units (RLUs). We analyzed them for relationship to cognitive function, CD4 cell counts and HIV viral load. Anti-Tat antibody levels were higher in those without neurocognitive dysfunction than in those with HIV-associated neurocognitive dysfunction (HAND), and in individuals with lower CD4 cell counts and higher viral loads. We provide details of an assay which may have diagnostic, prognostic, or therapeutic implications for patients with HAND. Active viral replication may be needed to drive the immune response against Tat protein, but this robust immune response against the protein may be neuroprotective.

### Keywords

neurotoxicity; neurovirology; dementia; neuroprotection; glutamate; neutralizing antibodies

### INTRODUCTION

HIV infects glial cells and can establish a reservoir within the brain. Infected cells in the brain can release neurotoxic viral proteins, including the HIV transactivator protein, Tat (Tornatore et al. 1994a; Tornatore et al. 1994b). The advent of highly active antiretroviral therapy (HAART) has successfully decreased the incidence of HIV dementia by lowering viral load, thus enabling the recovery of CD4+ T cells and restoring immunity (Sacktor et al. 2001). However, the prevalence of HIV-associated neurocognitive disorders (HAND) is actually increasing, because individuals on HAART are living longer (Dore et al. 2003; Neuenburg et al. 2002; Sacktor 2002; Sacktor et al. 2002; Simpson et al. 2003). Once proviral DNA is formed, HAART fails to target the early viral proteins. Of these proteins,

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Tat is released extracellularly, where it is highly neurotoxic and can be transported along neuronal pathways to cause neuronal injury at distant sites. It interacts with glial cells to induce activation and release of neurotoxic factors, leading to widespread damage. (Rumbaugh and Nath 2006).

Interestingly, some individuals never develop HAND despite severe immune suppression and high viral loads. This suggests that host susceptibility genes play an important role in the neuropathogenesis of this condition. The impact of variations in chemokines and cytokines likely reflects the importance of a host-generated inflammatory cascade (Gonzalez et al. 2002; Quasney et al. 2001). Another possibility is that variations in the host's adaptive immune response affect susceptibility to HAND. For example, the ability of the host to produce neutralizing antibodies to Tat may affect Tat's ability to cause neurotoxicity. This may have important implications for development of a vaccine strategy to treat or prevent HIV dementia and other neurological complications of HIV infection.

It is therefore imperative to monitor levels of antibodies to viral proteins within the CNS, as both diagnostic and prognostic markers. HIV infection is unique in that detection of virus or antibody to virus in the spinal fluid is not considered diagnostic of viral invasion of the CNS, whereas diagnosis of other common viral encephalitides, such as Herpes simplex encephalitis, require detection of virus or virus specific antibodies within the CNS. Previous studies have attempted to detect viral proteins and their transcripts within the CNS of infected individuals, but have not proven effective for quantitation due to lack of sensitivity (Hudson et al. 2000; Kruman et al. 1999; Trujillo et al. 1996; Westendorp et al. 1995; Wiley et al. 1996). Tat antibodies have not previously been studied in CSF.

## MATERIALS AND METHODS

### Study samples

CSF samples used were obtained from Northeastern AIDS Dementia (NEAD) cohort, Johns Hopkins Oxidative Stress cohort, and National NeuroAIDS Tissue Consortium (NNTC). Viral loads and CD4 cell counts were assessed at the same time that the cognitive testing was performed, as described (McArthur et al. 2004). HIV-infected samples were from individuals categorized by Memorial Sloan Kettering (MSK) scale as individuals with no cognitive impairment (MSK=0, n=15), with mild cognitive impairment (MSK=0.5–1, n=20), and with moderate cognitive impairment (MSK = 2, n=17). The HIV-infected individuals in this study showed no correlation between their neurocognitive status and their blood and CSF viral loads and CD4 cell counts. Thus, correlations which we demonstrate based on neurocognitive status should not be confounded by viral load or immune status.

Control groups included individuals with other neuro-inflammatory or neuro-infectious diseases (n=8) and non-inflammatory controls (normal pressure hydrocephalus, n=5). These individuals did not have risk factors for HIV infection. All samples had been centrifuged prior to storage, hence only cell free CSF was used. Table 1 provides a summary of clinical details of HIV-infected individuals. Table 2 provides clinical data regarding controls. Data on each individual patient is available in Table 3.

Use of the specimens required for this study was approved by the Johns Hopkins Institutional Review Board.

### Preparation of the ELISA Plate

A white opaque 96-well maxisorp plate (Nunc) was coated with Tat<sub>1-72</sub> at 2µg/ml in carbonate buffer (Na<sub>2</sub>CO<sub>3</sub> 1.59g/l, NaHCO<sub>3</sub> 2.93g/l, pH 9.6) and incubated overnight at 4°C. Recombinant Tat<sub>1-72</sub> was produced in our laboratory, as published previously (Ma and

Nath 1997; Turchan et al. 2001). The plate was then treated with blocking buffer (1% bovine serum albumin/0.1% Tween-20 in phosphate buffered saline [PBS], pH 7.4) for 2 hours at room temperature. Anti-tat antibodies were then added at 100 $\mu$ l/well serially diluted 10-fold in blocking buffer to generate standard curves with concentrations as low as 10<sup>-3</sup> pg/ $\mu$ l. We used either affinity-purified rabbit anti-Tat polyclonal antibodies (pABs) to whole Tat (generated in our laboratory) or mouse anti-Tat monoclonal antibody (mAB) to the C-terminus that was purified from a hybridoma cell line (NIH AIDS Research and Reference Reagent Program, Bethesda, MD, USA; cat#7383).

After the antibodies were incubated for 2 hours at room temperature, the plate was washed 3 times with washing buffer (0.1% Tween-20 in PBS, pH 7.4). Biotinylated antibodies, 100 $\mu$ l/well, diluted 1000-fold in blocking buffer, were incubated for 1 hour at room temperature: biotinylated goat anti-mouse antibody (Chemicon cat#AP130B) was used for wells incubated with mouse monoclonal antibodies and biotinylated goat anti-rabbit antibody (Chemicon cat#AP132B) for wells incubated with rabbit polyclonal antibodies. The plate was washed thereafter and streptavidin-HRP (BioRad cat#170-6528), 100 $\mu$ l/well, also diluted 1000-fold in blocking buffer, was incubated for 1 hour at room temperature. The plate was washed again and subsequently developed with SuperSignal Femto Sensitivity substrate (Pierce cat#37074), 100  $\mu$ l/well. The luminescence was measured within 5 minutes at 425 nm on a microplate reader (SpectraMax, Molecular Devices). These antibodies were found to have higher titer binding values, demonstrating optimal coating of Tat<sub>1-72</sub> to the plate.

### Treatment of CSF Samples

CSF samples were first heat-inactivated at 60°C for 30 minutes and then pre-treated with protease inhibitor cocktail (Sigma cat#P8340), 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM ethylene glycol tetraacetic acid (EGTA) to prevent non-specific interactions, stabilize the proteins, and disrupt the immune complexes.

### Detection of anti-Tat Antibody

CSF sample was added, in duplicate at 100  $\mu$ l/well and compared against normal CSF. After a 2-hour room temperature incubation, the plates were washed 3 times with washing buffer (0.1% v/v Tween-20 in PBS, pH 7.4). The plates were then incubated for 1 hour at room temperature with biotinylated anti-human antibodies (Chemicon cat#AP114B), diluted 1000-fold each in blocking buffer (1% w/v bovine serum albumin/0.1% v/v Tween-20 in phosphate buffered saline [PBS], pH 7.4). Wash steps were repeated, following which streptavidin-horse radish peroxidase (HRP), diluted 1000-fold in blocking buffer, was incubated on each plate for 1 hour at room temperature. The plates were washed again thereafter and analyzed using the SuperSignal Femto Sensitivity substrate (Pierce cat#37074). The luminescence was measured within 5 minutes at 425 nm on a microplate reader (SpectraMax, Molecular Devices).

### Statistical Analysis

To determine relative levels of anti-Tat antibodies in individuals' samples, the relative light units of HIV+ CSF samples were normalized against the RLU obtained from noninflammatory control CSF, used as a blank. Each plate had its own blank to account for plate-to-plate variability. "Undetectable" anti-Tat levels were defined as data points in the HIV positive groups that were less than the mean plus one standard deviation of the HIV negative controls. All data were analyzed using GraphPad Prism 5 by ANOVA using Newman-Keuls post-test comparison.

## RESULTS

The biotin-avidin based chemiluminescent ELISA has enabled us to achieve excellent sensitivity and specificity, spanning a broad range of detection from 10,000 to over 100,000 RLU for the anti-Tat antibodies in HIV-infected individuals (see figure 1).

### Detection of antibodies to Tat

CSF anti-Tat antibody levels were significantly higher in HIV-infected individuals without HAND compared to individuals with HAND (figure 2a). Highest levels of antibodies were present in HIV-infected patients with normal cognition. 3/15 had levels >70,000 RLU while none of the patients with mild impairment and only 1/37 with HAND had such high levels.

Next, we determined if CSF anti-Tat levels might depend on HIV RNA viral load in either serum (figure 2b) or CSF (figure 2c). Using a cutoff of 400 copies/ml, individuals with higher viral loads have significantly higher anti-Tat levels. Similarly, we hypothesized that CSF anti-Tat levels might vary with CD4 count. (Figure 2d) demonstrates that individuals with less than 250 CD4 lymphocytes/ $\mu$ l have significantly higher anti-Tat levels than those who are not as immunocompromised.

## DISCUSSION

HIV Tat has been strongly implicated as a cause of neurotoxicity in individuals with HAND. We therefore hypothesized that levels of anti-Tat in the spinal fluid might have a relationship with presence of HAND. We have hypothesized that anti-Tat antibodies neutralize Tat function, so that patients with high antibody levels might have less severe dementia. The data in figure 2a supports this hypothesis, indicating that CSF levels of anti-Tat antibody are inversely correlated with HAND severity. These results are also consistent with the neuroprotective properties of this antibody seen in *in vitro* studies (Rumbaugh et al. 2012).

Our ability to develop and apply quantitative chemiluminescent indirect ELISAs depended on a number of variables. A chemiluminescent end-point has increased sensitivity compared to using a colorimetric end-point. Anti-Tat detection was further optimized by pre-treating the CSF to stabilize the free proteins. Future studies might further demonstrate the specificity of this assay through use of blocking antigen.

Antibodies are typically thought of as marking toxins, bacteria, or infected cells for destruction by effectors such as complement molecules or phagocytic cells. Antibodies have also been shown to neutralize these toxic or infectious processes by binding to antigenic determinants on the harmful agent, thereby hindering the interaction of the agent with its receptor. One of the major proposed mechanisms of Tat-mediated neurotoxicity is binding and stimulation of NMDA receptors by Tat, producing excitotoxicity (Haughey et al. 2001; Prendergast et al. 2002; Self et al. 2004). Perhaps, the neuroprotective ability of an anti-Tat antibody is dependent on its ability to interfere with Tat's interaction with glial cells and Tat-mediated excitotoxicity.

HIV enters the brain within days to weeks of infection, establishes restricted infection in astrocytes and a productive infection in microglia/macrophages (Davis et al. 1992; Mankowski et al. 2002; Resnick et al. 1988). It may be years before individuals develop any dementing signs or symptoms, or they may never develop dementia at all (Davis et al. 1992; Mankowski et al. 2002; Resnick et al. 1988), suggesting a host susceptibility factor as a key

determinant. At least partly, this may be due to the presence or absence of an effective anti-Tat immune response.

It may seem counterintuitive that neuroprotective anti-Tat antibodies are higher in patients with lower CD4 counts, which are often the same patients who have neurocognitive dysfunction. However, in our cohort, there was no correlation between CD4 count and neurocognitive status. Thus, the correlation between high anti-Tat and low CD4 count in this study may be taken to mean that even patients with a low CD4 count can, at least in some cases, mount a humoral response to Tat, which may, in fact, help to explain why, in this cohort, no correlation was seen between CD4 count and neurocognitive status. Antibody levels to Tat were also higher in individuals with higher viral load. This may indicate that active Tat production is necessary for driving the antibody response, and it is possible that the antibody response may be an indirect measure for Tat production, which has been technically challenging to measure at low concentrations.

Future work should include individuals screened at regular intervals in order to assess whether baseline CSF anti-Tat levels can predict subsequent development of HAND, or whether these levels vary significantly in a single individual over a longitudinal timeframe. Such work should also carefully scrutinize the HAART regimens of individuals to determine whether more or less CSF penetrating regimens correlate with higher or lower CSF anti-Tat levels. Such a longitudinal study could also assess whether changes in anti-Tat levels that may become evident over time, perhaps associated with changes in HAART regimen, can predict a better prognosis.

The ability to detect anti-Tat antibody levels in the CSF by a relatively easy ELISA technique as described here may also have important implications for therapeutic development to treat or prevent HAND. One possible treatment would be development of a vaccine strategy or perhaps the use of a therapeutic monoclonal antibody against Tat. Efficient development of such a therapy would require the ability to easily monitor anti-Tat antibodies in the CSF.

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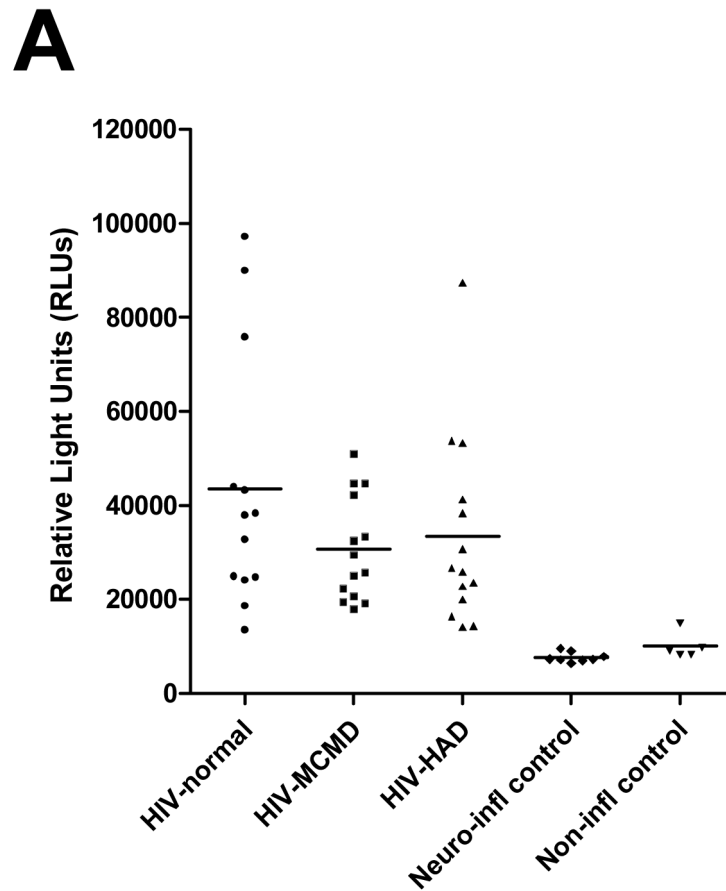
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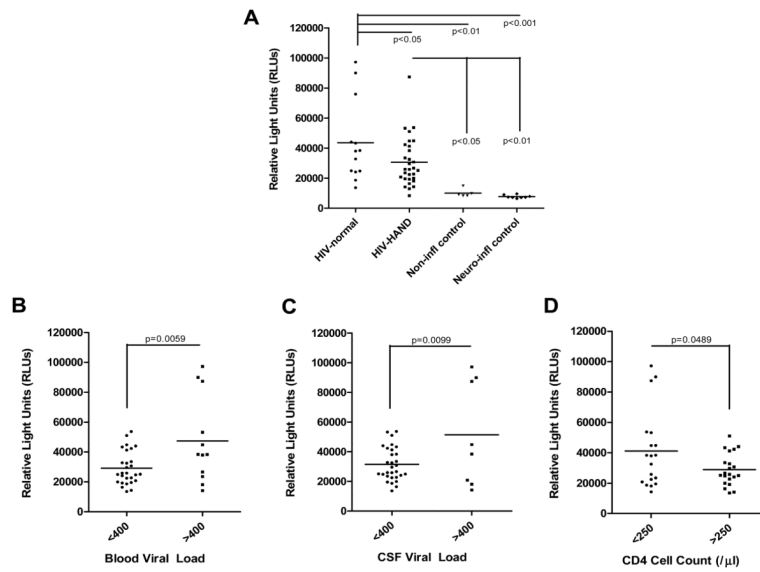
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**Fig 1.** Relative Light Units of an ELISA for anti-Tat antibodies detected in spinal fluid of HIV-infected individuals without dementia (HIV-Normal) (MSK 0) (n=15; 86.7% > mean+1SD), with mild cognitive impairment (HIV-MCMD) (MSK 0.5-1) (n=20; 70% > mean+1SD), with moderate cognitive impairment (HIV-HAD) (MSK 2) (n=17; 82.4% > mean+1SD), HIV negative controls (n=5), and neuro-inflammatory controls (n=8). Anti-Tat levels in spinal fluid of all HIV groups are significantly higher than in controls. The points in the figure represent individual patient data and lines represent the mean. Statistical analysis was by ANOVA, with Newman-Keuls post-test.



**Fig 2.**

Detection of Antibodies to Tat in the CSF of individuals infected with HIV: (A) Relative Light Units of an ELISA for anti-Tat antibodies detected in spinal fluid of HIV-infected individuals without dementia (HIV-Normal) (MSK 0) (n=15; 86.7% >mean+1SD), with HIV-associated neurocognitive disorder (HAND) (MSK 0.5) (n=37; 83.8% >mean+1SD), HIV negative controls (n=5), and neuro-inflammatory controls (n=8). Anti-Tat levels in spinal fluid of the HIV-Normal group were significantly higher than in HIV-HAND ( $p<0.05$ ). Antibody level was significantly higher in HIV-infected individuals without dementia than the HIV negative individuals ( $p<0.01$ ) and the neuro-inflammatory control individuals ( $p<0.001$ ). CSF anti-Tat levels are higher in individuals with higher serum and CSF viral loads and lower CD4 cell counts. (B) In the CSF of individuals with HIV (n=51), anti-Tat levels are significantly higher in the group with serum viral load >400 copies/ml ( $p<0.01$ ). (C) In the CSF of individuals with HIV (n=47), anti-Tat levels are significantly higher in the group with CSF viral load >400 copies/ml ( $p<0.01$ ). (D) In the CSF of individuals with HIV (n=51), anti-Tat levels are significantly higher in the group with CD4 cell level <250 cells/μl ( $p<0.05$ ). In all panels, the points in the figure represent individual patient data and lines represent the mean. Statistical analysis was by ANOVA with Newman-Keuls post-test for (A) and by unpaired t-test for (B, C, D).

**Table 1**

Summary of the clinical data of HIV-infected individuals used in this study: HIV infected individuals without dementia (HIV+ normal) (MSK 0) (n=15), with mild cognitive impairment (MSK 0.5-1) (n=20), with moderate cognitive impairment (MSK 2) (n=17). Blood and CSF viral loads represent copies/ml and CD4 cells represent lymphocyte cell count per  $\mu$ l.

	age (median)	SEX (n)		blood viral load (n)		CSF viral load (n)			CD4			
		male	female	<400	>400	<400	>400	unavailable	average	median	<250 (n)	>250 (n)
HIV+ Normal	47.5	9	5	9	5	11	3	0	372.8	319.5	6	8
HIV+ mild cog impair	45.5	15	5	15	5	14	3	3	297.8	268.5	9	11
HIV+ mod cog impair	47	14	3	10	7	14	2	1	347.1	320	10	7

**Table 2**

Summary of the clinical diagnosis of HIV-negative individuals used in this study: Neuro-inflammatory control individuals (n=8) and non-inflammatory control individuals (n=5).

	Sex (n)		Diagnosis (n)			
	Male	Female	Neurosar-coidosis	Multiple Sclerosis	Transverse Myelitis/Multiple Sclerosis	Normal Pressure Hydrocephalus
Neuro-Inflammatory	5	3	2	5	1	0
Non-Inflammatory	3	2	0	0	0	5

**Table 3**

Clinical data of HIV-infected individuals used in this study: HIV infected individuals without dementia (HIV-no dementia) (MSK 0) (n=15), with mild cognitive impairment (MSK 0.5-1) (n=20), with moderate cognitive impairment (MSK 2) (n=17). Blood and CSF viral loads represent copies/ml and CD4 cells represent lymphocyte cell count per  $\mu$ l.

	age	sex	Yr HIV inf	Blood VL	CSF VL	CD4	date of death
<b>HIV-Normal</b>	61	F	1994	ND	ND	419	
	48	M	1988	17629	17290	101	4/14/2006
	48	M	1988	<400	ND	1113	
	47	M	1981	20916	507	36	
	33	F	1992	14280	434	152	
	46	F	1989	<50	ND	418	
	62	M	1988	<50	ND	651	
	66	M	1992	<50	ND	150	
	44	M	1991	1805	<50	354	
	41	F	1994	<50	<50	663	
	54	M	1995	<50	<50	285	
	52	F	1995	<50	<50	618	
	37	M	2001	32256	<50	12	
	31	M	2005	<50	<50	247	
	<b>HIV-mild cog impair</b>	57	M	1990	ND	ND	405
57		M	1989	ND	ND	221	2/19/2006
40		M	1993	<400	ND	189	
41		M	1991	<400	1399	202	
49		M	1996	<400	<50	170	
52		M	1992	<50	ND	250	
62		M	1988	<50	unavail	287	
45		F	1991	66328	2869	2	
45		M	1995	13586	unavail	48	
42		M	1986	<400	<400	460	
49		M	2001	<50	<50	543	
42		F	1992	<50	<50	617	

age	sex	Yr HIV inf	Blood VL	CSF VL	CD4	date of death
49	M	1993	21777	3072	204	
55	F	1994	<50	<50	455	
50	M	1989	<50	unavail	490	
46	F	1993	<50	<50	173	
38	M	1985	>100000	97	5	
44	M	2004	<50	<50	302	
45	M	1992	<50	<50	494	
37	F	1996	5538	<50	439	
<b>HIV-mod cog impair</b>						
50	M	1986	<400	<50	983	
41	M	1992	29381	ND	15	5/22/2004
44	M	1996	38207	9656	28	
45	M	1997	170352	ND	165	
61	M	1985	1096	ND	57	
51	M	1992	<50	ND	665	
49	F	1995	<50	ND	671	
46	M	1998	<50	ND	320	
42	F	1992	928	161	309	
46	M	1986	221756	1618	395	
46	M	1990	<50	<50	422	
55	M	1985	285	unavail	135	
47	M	1997	83	<50	417	
51	M	1989	65836	336	2	
34	M	1999	<50	<50	520	
54	F	1988	<50	<50	215	
59	M	1986	<50	<50	582	