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Adhesion of Human Neuroblasts to HIV-1 *tat*

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ABSTRACT

Several neuropathologic findings in infants and children with human immunodeficiency virus type-1 (HIV-1) infection are different from those observed in adults, probably related to the fact that the retroviral infection occurs in the setting of neurodevelopment. This report describes the interaction and biologic activity of *tat*, the HIV-1 *trans*-activating protein on human neuroblasts. Two human neuroblastoma cell lines, LAN-5 and GI-CA-N, have been studied for their capability to adhere to *tat* (full recombinant protein) and to two different peptide residues of it. Both cells adhere to *tat* and tat^{46-60} basic domain, although not to tat^{65-80} residue, which contains the RGD (arginine-glycine-aspartic acid) motif. Adhesion to collagen I was inhibited by preincubating GI-CA-N cells with $tat,^{46-60}$ although not with $tat,^{65-80}$ indicating the capability of the basic residue to interfere with collagen I-induced cellular adhesion. The expression of 200-kD neurofilaments induced by collagen I was not induced by $tat,^{46-60}$ indicating that neural differentiation along the same pathway is not mimicked by this peptide. Neuroblast cell proliferation was not affected by adhesion to tat^{46-60} nor to $tat.^{65-80}$ GI-CA-N cells are not permissive to HIV-1 infection. However, proviral DNA was documented in the cell lysate for 14 consecutive *in vitro* passages, whereas HIV-1 transcription was never detectable. This would exclude the possibility that *tat* would be transduced by these cells. GI-CA-N stained negative for CD4, although positive for Gal-C, which may explain HIV-1 entry. Results show that immature human neural cells interact with *tat* protein and/or its basic residue *in vitro*. A mechanism similar to that herein described would possibly be active *in vivo*, which may help in clarifying the pathogenic mechanisms of neurologic dysfunction and destruction of the CNS observed in infants infected with HIV-1. (***Pediatr Res* 38: 792–796, 1995**)

Abbreviations

HIV-1, human immunodeficiency virus type 1
ECM, extracellular matrix
TNF-α, tumor necrosis factor-α
γ-IFN, γ-interferon
TGF-β1, transforming growth factor-β1
NB, human neuroblastoma
PCR, polymerase chain reaction
Coll, collagen
RT, reverse transcriptase
B-LCL, B lymphoblastoid cell line
VIP, vasoactive intestinal peptide

HIV-1, the etiologic agent of AIDS, is often complicated with neurologic disorders (1). HIV-1-associated neurologic disease occurs as the initial presenting clinical manifestation of AIDS in 3–7% of infected patients, but in up to 18% of children (2). In infants infected with HIV-1, the CNS is involved earlier than in adults, partly related to the fact that the retroviral infection occurs in the setting of neurodevelopment (3). Intrinsic effects of HIV-1 on the infant's CNS include microcephaly, diffuse gliosis, and basal ganglia mineralization (3). In addition, an intrinsic HIV-1-induced damage has been indirectly demonstrated by the successful treatment of various forms of dementia and neurologic symptoms with zidovudine (4–6). Moreover, HIV-infected children have altered cortisol secretion, probably associated with specific CNS damage (7). The pathogenic mechanisms of neurologic dysfunction and destruction—whether a result of direct cellular infection of HIV, secondarily produced and up-regulated cytotoxic cytokines, or co-infection with opportunistic pathogens—remain an area of active research. During early human brain development, HIV-1 may influence the differentiation processes of $CD4^-$ neuroblasts and oligodendrocyte precursors, resulting in dysmyelination and gliosis (1–7).

Neural cell adhesion to ECM protein is a crucial step in both neural cell differentiation and function (8–16). We recently showed that agents affecting neuroblast differentiation, such as retinoic acid, TNF-α, and γ-IFN, modulate NB cell differentiation; this includes integrin receptor expression (17) and induction of HLA class II antigen (18). Thus, changes in NB cell-adhesion properties are part of the maturational processes induced by the various differentiators (13,14). In the nervous system integrins are the major class of adhesion receptors and mediate the differentiating effects of ECM components on neural cells during development (7–18).

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The HIV-1 *trans*-activator protein *tat* has been implicated as a mediator of neuronal dysfunction in several model systems (19–24). In a recent report, Kolson *et al.* (23) showed that *tat* induced a marked aggregation of neurons and astrocytes in primary rodent brain cell cultures and caused the neuritic processes to coalesce into fascicles, thus affecting neural cell adhesion processes. These effects mapped to a different region from the *trans*-activation domain of *tat*, as mutating the RGD sequence within the second exon abrogated aggregation and fascicle formation without affecting *trans*-activation capacity. PC12, a rat pheocromocytoma-derived cell line, would also be affected by *tat* in their differentiation/proliferation processes (19).

Proliferation and differentiation of human cells other than neural cells is affected by *tat*. By studying spindle-shaped cells of vascular origin, that are the probable tumor cells of Kaposi sarcoma, Barillari *et al.* (24) showed that *tat* promotes the adhesion of AIDS-Kaposi sarcoma as well as normal vascular cells. Such an adhesion is associated with the amino acid sequence RGD of *tat* through a specific interaction with the integrin receptors $\alpha5\beta1$ and $\alpha(v)\beta3$, although it is augmented by the basic region, the 46–60 domain. Moreover, the expression of both integrins is increased by cytokines promoting these cells to acquire spindle morphology and become responsive to the adhesion and growth effects of *tat*. Barillari's results strongly suggest that RGD-recognizing integrins mediate both adhesion and vascular cell growth-promoting effect of *tat*.

By studying the modulation of integrin receptors induced by γ-IFN in human NB cells, we previously showed a specific modulation of $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ along with the neural differentiation process (15). In this report we analyze the adhesion, proliferation, and morphologic changes due to HIV-1 *tat* and discuss the possible implication of neural cell adhesion disturbance by *tat* in the etiology of CNS damage by HIV-1.

METHODS

Chemicals. Human ECM, including laminin, fibronectin, Coll I, Coll IV, and vitronectin were purchased from Sigma (St Louis, MO). *tat*-derived peptides were produced by Neosystem (Strasbourg, France); tat^{46-60} was synthesized according to the following sequence: S-Y-G-R-K-K-R-R-P-P-Q, whereas the tat^{65-80} sequence was: H-Q-V-S-L-S-K-Q-P-T-S-Q-P-R-G-D. Poly-D-lysine was purchased from Polyscience Inc. (Warrington PA). FCS, BSA and cell culture media were all from Seromed. VIP and substance P were both from Sigma.

Cell lines. LAN-5 NB cell line was a generous gift of R. Seeger (UCLA), whereas the GI-CA-N cell line was established in our laboratory as described elsewhere (26).

Cell adhesion. Cell lines and methods have been previously described in detail (15, 16, 26–29). Briefly, GI-CA-N and LAN-5 cells were harvested with PBS, resuspended in RPMI 1640, and seeded on 96-well plates (Costar, Cambridge MA) previously coated with ECM substrates or *tat* residues (10 μg/ml final concentration on plastic plates preactivated with *bis*-bromo-sulfosuccynimidyl-suberate, BS3, Pierce). Poly-D-lysine was used as a control. Coating was performed overnight at 4°C, then the plates were washed twice with PBS and incubated for 1 h at room temperature with 0.1% BSA in PBS. After two additional washes, cells were plated and incubated for 1 h at 37°C. Unattached cells were washed out with PBS, whereas adherent cells were fixed and stained with cresyl violet in 20% methanol. After solubilization with 0.1 M citric acid in 50% ethanol, A_{600} was measured by microplate reader Metertech, model Σ960. The percentage of cells attached to ECM, poly-D-lysine and *tat* peptides was calculated as described elsewhere (15).

To evaluate whether *tat* fragments were able to compete with ECM proteins, preincubation of both cell lines with *tat*-derived peptides was performed. Cells were preincubated for 1 h at 37°C with tat^{46-60} or to tat^{65-80} at the concentration of 100 μg/ml, washed, and tested in the adhesion assay. A preincubation with control peptides, including VIP and substance P, was performed under the same conditions. To test whether Coll I receptor was involved in tat^{46-60} recognition, a preincubation with soluble Coll I was performed for 1 h at 37°C. Cells were then washed and tested in the adhesion assay.

Integrin pattern. The expression of integrin receptors was evaluated by means of immunoprecipitation and/or RT-PCR as recently described in detail (15).

Cell proliferation and morphology. NB cells proliferation was evaluated by [^{3}H]TdR incorporation as described before in detail (23). Morphologic and immunophenotype changes were also evaluated by mean of immunocytochemistry as described elsewhere (15, 16, 26–29). Briefly, the percent of positive cells was evaluated by two different researchers, blind to study senses. Data were expressed as the mean ± SD of four different experiments (see Table 2 legend for further details).

Proliferation of NB cells was also tested on 96-well plates precoated with *tat* residues and control peptides, including substance P and BSA.

HIV-1 infection. Infection with HIV-1 was obtained by co-cultiring GI-CA-N with a B-LCL chronically infected with HIV-1_{IIIB}, as described elsewhere (30). Briefly, B-LCL cells obtained by transformation of peripheral blood lymphocytes with EBV were acutely infected with HIV-1_{IIIB} (30). To this end, 1×10^6 B-LCL were incubated with 100 μl of stock HIV-1_{IIIB} supernatant in a final volume of 1 ml corresponding to a multiplicity of infection of approximately 0.01. After incubation at 37°C for 32 h, the cells were extensively washed and cultured in T50 cell culture flasks in RPMI 1640 (Life Technologies, Inc.) + 10% FCS (ICN-Flow).

The time course of infection was monitored by the determination of HIVp24 antigen appearance in supernatants. On d 7 postinfection, when virus replication reached approximately 50% of peak levels, the HIV-1-infected B-LCL were washed extensively and γ-irradiated (7000 rad). After further washing, 10^5 B-LCL infected with HIV-1_{IIIB} were co-cultured with 5×10^5 GI-CA-N cells that had been plated 24 h previously in duplicate wells of a 24-well plate (Costar).

The cultures were split after complete confluence (approximately every 4–6 d) and periodically monitored for infection. The relative cell composition of the cultures was monitored by cell fluorimetry using FITC-conjugated MAb that are expressed on B-CLC, including CD4, CD45R0, and CD19. Infection of surviving GI-CA-N cells was assessed by detection

of HIV-1 p24 antigen in the culture supernatant and by detection of proviral DNA sequences by PCR as follows.

PCR analysis. HIV-1 proviral DNA was detected according to Schnittman *et al.* (31). Briefly, at passages 3, 5, and 7 after infection, GI-CA-N cells were washed and pelleted in PBS, lysed in 0.001% Triton X-100/0.0001% SDS in TDE buffer (10 mM Tris-HCl pH 8.0, 0.5 mM EDTA, pH 8) with 600 μg/ml proteinase K (Boehringer Manheim, Indianapolis, IN) for 1 h at 56°C, 15 min, at 95°C. The PCR reaction mixture contained 50 μl of DNA lysate, 50 pmol of each primer, SK38/SK39 (gag 1551–1578; gag 1638–1665), 200 μM each of four deoxynucleotide triphosphates (Boehringer Manheim), 10 mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.2% gelatin, and 2 U of DNA polymerase from *Thermus aquaticus* (Perkin-Elmer Corp.)

Amplification was carried out with a Perkin-Elmer thermocycler 480 (denaturation 94°C, annealing 55°C, extension 72°C, 30 cycles). After amplification, 30-μl aliquots were mixed with ^{32}P-labeled SK19 probe, denatured at 94°C, and hybridized at 56°C for 15 min. Autoradiograms of polyacrylamide gels were obtained by exposure to Kodak XAR film at −70°C (see Fig. 3).

RESULTS

GI-CA-N cells adhere to all ECM tested, whereas LAN-5 cells attached to Coll I, Coll IV, and laminin but showed very weak adhesion to vitronectin and fibronectin. Both cells were capable of adhering to tat^{46-60}, although not to tat^{65-80} (Fig. 1). In spite of this both cell lines were able to grow on plastic dishes precoated with either residue (Table 1).

Results related to competition experiments are reported in Figure 2, *A* and *B*. Inhibition of cell adhesion was evaluated by comparing optical densities. Preincubation of GI-CA-N cells with soluble tat^{46-60}, but not soluble tat^{65-80}, inhibits the attachment of cells to a matrix of Coll I (Fig. 2*A*). In the same way, challenging the cells with soluble Coll I inhibits the attachment to a matrix of tat^{46-60} (Fig. 2*B*), thus suggesting the presence of a common ligand for both proteins on the surface of GI-CA-N.

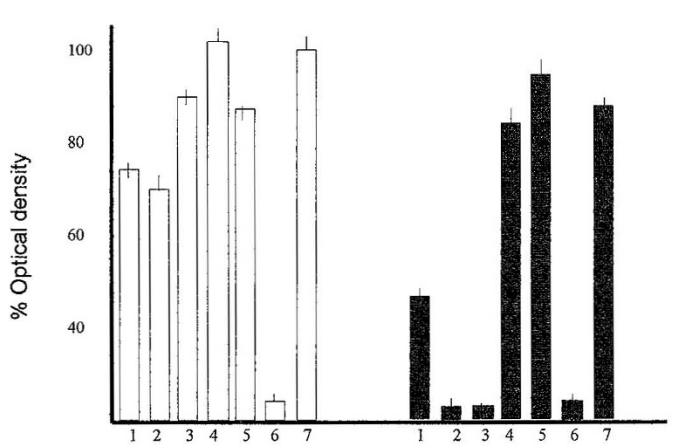

Figure 1. Adhesion of GI-CA-N (□) and LAN-5 (■) cells to ECM proteins and HIV-1 *tat* residues. *1*, laminin; *2*, Vitronectin; *3*, fibronectin; *4*, Coll I; *5*, Coll IV; *6*, tat^{65-80}; *7*,tat^{46-60}.

Table 1. *Adhesion of Gi-Ca-N and LAN-5 to ECM protein and or HIV-1 tat*

Adhesion to*	GI-CA-N	LAN-5
BSA	0.2–0.27	0.15–1.151
Coll I	0.7–0.56	0.388–0.4
FN	0.23–0.24	0.823–0.9
tat	0.94–0.95	0.416–0.4
tat^{46-60}	0.6–0.751	0.2–0.217
tat^{65-80}	0.136–0.169	0.367–0.373

Cells were seeded on precoated slides, allowed to adhere for 90 min at 37°C, fixed and stained, and evaluated as A_{600} by microplate reader as described in Methods. Results are expressed as range obtained in three different experiments performed in duplicate. SD has been omitted being in each case < 10%.

* BSA (blank); tat^{46-60} and tat^{65-80}, synthetic peptides respectively from tat basic residue and RGD-containing motif.

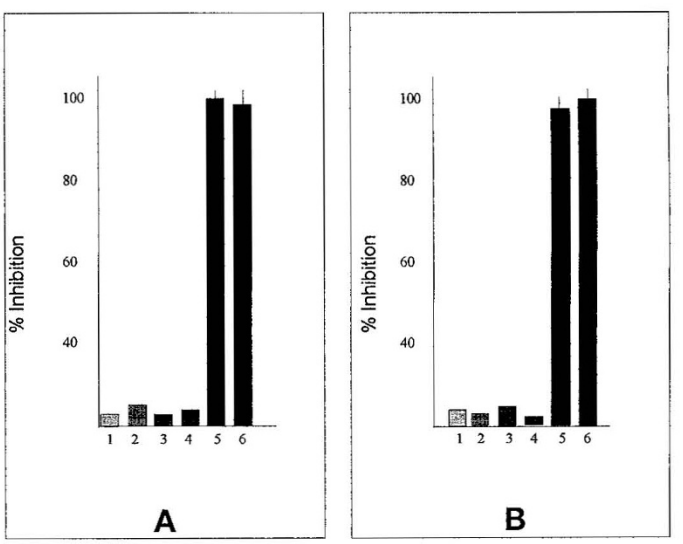

Figure 2. Inhibition of adhesion to Coll I and tat^{46-60} by preincubating GI-CA-N cells with tat^{46-60}, Coll I and control peptides. (*A*) Adhesion to Coll I; (*B*) adhesion to tat^{46-60}. Gi-CA-N human neuroblastoma cells have been tested as following: *1*, preincubation with RPMI-1640; *2*, preincubation with BSA; *3*, preincubation with tat^{65-80}; *4*, preincubation with Substance P; *5*, preincubation with Coll I; *6*, preincubation with tat^{46-60}.

GI-CA-N cells were positive for Gal-C, particularly when seeded on tat^{46-60}; the expression of 200-kD neurofilaments was induced by allowing cells to adhere on Coll I. Such an expression was not observed in cells adhering to tat^{46-60} (Table 2). Cell proliferation was not significantly inhibited by culturing LAN5 or GI-CA-N cells on either *tat* residues (not shown). When cultured on Coll I, both LAN-5 and GI-CA-N cells acquired a neuronal morphology (extension of neurite-like processes and filipodia), whereas cells adhering to tat^{46-60} were small, round, and often clustering as pseudo-rosettes.

The expression of integrin subunits by both GI-CA-N and LAN-5 cells is summarized in Table 3. As expected from the adhesion results, the pattern of the two cell lines was different. Both expressed β_1 when associated with different α isoforms.

Both GI-CA-N and LAN-5 cells were tested as negative for CD4, confirming previously published results (6). Figure 3 shows the gradual loss of HIV-1 DNA-specific sequence as detected by PCR, in GI-CA-N/B-LCL co-culture.

Table 2. *Immunocytochemical pattern of GI-CA-N and LAN-5 cells seaded on Coll-I and tat*$^{46-60}$

	GI-CA-N			LAN-5		
MAb*	Control	Coll I	*tat*$^{46-60}$	Control	Coll I	*tat*$^{46-60}$
GAL-C	+†	±	+	Neg	Neg	Neg
HNK-1	+	+	+	+	+++	+++
GFAP	Neg	Neg	Neg	Neg	Neg	Neg
200-kD NF	±	+++	±	+	Neg	Neg
NGFr	+	Neg	Neg	±	Neg	Neg
N-CAM	+	+	+	+	±	Neg
MAG	Neg	Neg	Neg	Neg	Neg	Neg

Cells were seaded on precoated slides, allowed to adhere for 90 min at 37°C, fixed, and stained with a standard procedure, as described.

* Gal-C, galactosyl-cerebroside; HNK-1, human NK-1; GFAP, glyal fibrillar acidic protein; NF, neurofilaments; NGFr, nerve growth factor receptor; N-CAM, neural cell adhesion molecule; MAG, myelin-associated glycoprotein.

† Fluorescence intensity: (neg) negative; (±) equivocal; (+) weakly positive; (++) moderately positive; (+++) strongly positive. Antibodies used and specific methods have been previously specified in detail (8, 16, 17).

Table 3. *Expression of integrin subunits by GI-CA-N and LAN-5 human neuroblastoma cells*

	LAN-5		GI-CA-N	
Integrin subunits	RT-PCR	IP	RT-PCR	IP
β_1	Pos	Pos	Pos	Pos
α_1	Pos	Pos	Neg	Neg
α_2	Pos	Pos	Pos	Pos
α_3	Pos	Pos	Pos	Pos
α_4	Pos	Neg	Pos	Pos
α_5	Neg	Neg	Pos	Pos
α_{6A}	Pos	Neg	Pos	Pos
α_{6B}	Pos	Neg	Pos	Pos
α_v	Pos	Pos	Pos	Pos
β_3	Pos	Pos	Pos	ND
β_4	Pos	Neg	Neg	Neg
β_5	Neg	ND	Pos	ND
β_6	Neg	Neg	Neg	Neg

IP, immunoprecipitation; ND, not done (see Ref. 12 for details).

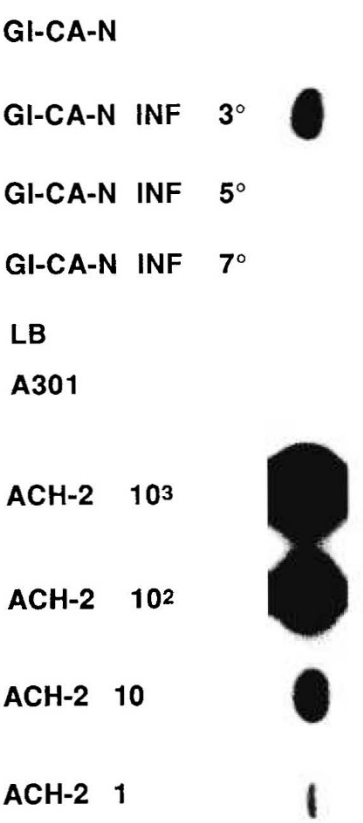

Figure 3. PCR amplification of HIV-1 DNA in uninfected and in infected GI-CA-N cells at pasages 3, 5, and 7 after infection. DNA amplification was obtained by using *gag*-specific primers as described in Methods. Intensities of PCR signal in 6×10^4 cells are compared with 10-fold serial dilutions of cell lysates from ACH-2 (positive control). *LB*, lysis buffer (negative control); *A301*, cells (uninfected negative control).

DISCUSSION

The absence of $\alpha 4$ and $\alpha 5$ subunits in LAN-5 cells explain the weaker adhesion to fibronectin, compared with GI-CA-N which express these isoforms. Because both cell lines firmly adhere to *tat*$^{46-60}$, although not to *tat*$^{65-80}$, the integrin receptors involved in fibronectin recognition which include $\alpha 4$ and $\alpha 5$ subunits should not be involved. Adhesion to *tat*$^{46-60}$ is inhibited by preincubating cells with Coll I (Fig. 2), although not with fibronectin nor with laminin (not shown). In addition, preincubation of cells with *tat*$^{46-60}$ inhibit attachment to the same fragment and to Coll I (Fig. 2), although not to the other ECM (not shown). Preincubation with VIP, substance P, and *tat*$^{65-80}$ did not inhibit adhesion to *tat*$^{46-60}$ nor to Coll I. This is partially in contrast with Barillari's report (24). By testing spindle-shaped vascular endothelial cells, these authors demonstrated that the adhesion to HIV-1 *tat* is associated with the amino acid sequence RGD through a specific interaction with the integrin receptors $\alpha_5\beta_1$ and $\alpha_v\beta_3$, although it is augmented by the basic region. In the neural cell model herein described, the implication of α_5 and RGD domain cannot be excluded for GI-CA-N, although it is excluded for LAN-5 (Table 3; Fig. 1). Thus, the role of the basic residue appears to be more than just accessory. It is then possible that human neural cells adhere to *tat* with receptors other than that identified for endothelial cells (24).

By infecting NB cells with HIV-1 we ascertained the lack of permissivity of GI-CA-N ($CD4,^-$ Gal-C^+) to the infection. Thus, GI-CA-N cells may possibly allow HIV-1 entry through Gal-C, although not permissive to HIV-1 replication. We do not know yet if infected cells may eventually transduce and secrete *tat*; experiments to determine this are presently being run in our laboratory. However, it is clear now that neural cells not permissive to HIV-1 infection may adhere to a *tat* residue which compete with Coll I. The effect of a three-dimensional Coll I preparation on the structural organization of GI-CA-N

cells has been recently observed in our laboratory (G. Alessandri, unpublished results). In this model, Coll I induces GI-CA-N cell growth arrest and neuronal differentiation. A similar phenomenon has been recently observed by Bergsteins-Dottir *et al.* (10) in a different neural cell model. Thus, it is likely that adhesion to *tat* may perturb the neuronal differentiation processes, including the inhibition of 200-kD NF expression (Table 2), by a mechanism probably based on Coll I receptor competition.

According to Ensoli *et al.* (33) HIV-1 *tat* is secreted by infected cells and possibly degraded by membrane and extracellular proteases to smaller fragments. By showing here that some of the *tat* fragments interact with ECM receptors, we confirm that a etiopathogenetic mechanism of neural cell function disruption may be based on such a phenomenon. Results presented by Cupp *et al.* (22) would confirm this hypothesis. These authors demonstrated the ability of the HIV-1 *tat* protein to increase expression of TGF-β^1, a cytokine with potent immunosuppressive activity, in human astrocytic glial cells. Thus, implications of the *tat*-mediated induction of TGF-β1 expression and cytokine involvement in the regulation of immune response and CNS pathology is very likely.

In previous reports we demonstrated that γ-IFN and TNF-α, both of which are actively secreted by HIV-1-infected cells, modulate human neural cell proliferation and differentiation (13,23,24), modifying integrin expression (12). Thus, *tat*, by altering a complex combination of cytokine regulation and adhesion processes, may affect neuron and accessory cell maturational processes. The involvement of *tat* in altering the expression of cellular regulatory factors, including ECM receptors which, in turn, may mediate the altered physiology of the cells would result in dysmyelination and gliosis. Other HIV-1 proteins may also be involved. Recent results in the study *in vivo* of a mice transgenic model support the evidence that gp120 also plays a key part in HIV-1-associated nervous system impairment (32). Thus, the interaction of gp120 and *tat* in determining the observed neurotoxicity in the developing nervous system is very likely.

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