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Inhibition of Antigen-Induced Lymphocyte Proliferation by Tat Protein from HIV-1

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The purified human immunodeficiency virus type–1 (HIV-1) Tat protein inhibited lymphocyte proliferation induced by tetanus toxoid or Candida antigens by 66 to 97% at nanomolar concentrations of Tat. In contrast, Tat did not cause a significant reduction of lymphocyte proliferation in response to mitogens such as phytohemagglutinin or pokeweed mitogen. Inhibition was blocked by oxidation of the cysteine-rich region of Tat or by incubation with an antibody to Tat before the assay. A synthetic Tat peptide (residues 1 to 58) also inhibited antigen-stimulated proliferation. Experiments with H9 and U937 cell lines showed that Tat can easily enter both lymphocytes and monocytes. The specific inhibition of antigen-induced lymphocyte proliferation by Tat mimics the effect seen with lymphocytes from HIV-infected individuals and suggests that Tat might directly contribute to the immunosuppression associated with HIV infection.

THE TAT PROTEIN FROM HUMAN IMmunodeficiency virus activates HIV-1 gene expression and is essential for viral replication in vitro (*1, 2*). Tat can be taken up by cells growing in tissue culture, enter the nucleus, and transactivate genes expressed from the HIV-1 promoter (*3*). This property of Tat raises questions about the biological importance of extracellular Tat during the course of HIV infection in vivo, but it also provides a simple way to study the effects of Tat in the absence of viral replication. To test whether Tat might play a direct role in immune dysfunction in addition to its role in viral replication, we measured the effect of extracellular Tat in lymphocyte proliferation assays. We now show that Tat inhibits antigen-induced, but not mitogen-induced, lymphocyte proliferation.

The Tat protein (amino acids 1 to 72) was expressed in *Escherichia coli* and purified to >95% homogeneity, as described (*3, 4*). Lyophilized Tat protein was resuspended at 200 μg/ml in distilled water containing 10 m*M* β-mercaptoethanol. A reducing agent was included to help prevent oxidation, which can lead to formation of disulfide-linked multimers and loss of activity (*4*). We assayed lymphocyte proliferation in the presence or absence of Tat protein. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy HIV-1 seronegative donors and incubated with or without Tat for 1 hour at 37°C. Soluble tetanus toxoid or Candida antigen was then added to the cultures. On day 6, cultures were pulsed with [^{3}H]thymidine and harvested. For mitogen stimulation, 5×10^4 cells were incubated with phytohemagglutinin-P (PHA), concanavalin A (Con A), or *Staphylococcus aureus* protein A (Sp A) for 3 days or with pokeweed mitogen (PWM) for 7 days.

Tat inhibited the proliferative response to tetanus toxoid by 66 to 97% and the response to Candida antigens by 75 to 91%. Representative data from three individuals are shown in Table 1. Differences in antigen-induced proliferation in the presence or absence of Tat were, in each case, statistically significant (range; $P < 0.0055$ to $P < 0.00001$; two-tailed t test). Four additional subjects have been tested, and all showed similar responses. The inhibitory activity of Tat was concentration dependent (Fig. 1). Tetanus toxoid–induced proliferation was inhibited 81% with Tat at 10 μg/ml and 50% with ~0.5 μg/ml. Only a single dose of Tat was needed during the 6-day incubation even though the half-life of the protein in tissue culture medium is less than 24 hours (*3*). The inhibition is unlikely to be due to a cytotoxic effect of the Tat

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preparation since PHA-induced proliferative responses were not inhibited (Table 1). Other mitogens (Con A, Sp A, and PWM) were also tested and showed no significant inhibition (*5*). Since PWM requires a 7-day treatment, inhibition cannot simply be due to long-term incubation with Tat. To determine at what time Tat was required for antigen-specific inhibition, we treated PBMC cultures with Tat for 1 hour and washed the cells before stimulation with tetanus. Lymphocyte proliferation was inhibited by 70%; however, when Tat was added to PBMC cultures 1 day after stimulation with tetanus, negligible inhibition was seen (3% inhibition).

Several experiments provided evidence that the immunosuppressive activity was due specifically to Tat. The Tat protein contains a cysteine-rich region that mediates metal-linked dimerization in vitro (*4, 6*) and is essential for its biological activity as a transactivator (*7*). Oxidation of the cysteines results in disulfide-linked multimers with decreased biological activity. To test the effect of oxidation, we bubbled 100% O_2 through a Tat protein sample at neutral *p*H, resulting in almost complete loss of inhibitory activity (Table 2). Incubation of Tat with a polyclonal rabbit antibody to Tat (anti-Tat), but not with preimmune serum, before addition to PBMC cultures resulted in the loss of inhibitory activity for tetanus toxoid–induced proliferative responses (Table 2). A different source of Tat was also tested for inhibition. A synthetic peptide containing Tat residues 1 to 58, which is active for transactivation (*8*), also inhibited tetanus-stimulated proliferation (Table 2). Concentrations of the peptide ten times as great as for Tat 1–72 were required to inhibit proliferation, correlating with its reduced activity as a transactivator (*8*). We also tested another Tat protein, which is encoded by the two exons of Tat (residues 1 to 86), to see if there was any functional difference between the two forms of the protein. Both proteins have similar transactivation activity (*8*). Bacterially expressed Tat 1–86 (0.4 μg per culture) (*3*) also inhibited tetanus-induced proliferation (55% inhibition) and had no effect on PHA-induced proliferation.

Because Tat is taken up by HeLa cells growing in tissue culture and transactivates the HIV-1 promoter (*3*), Tat might also directly enter lymphocytes or monocytes, and this might help explain the effect of Tat on lymphocyte proliferation. To test this, we treated H9 lymphoid and U937 promonocytic cells that have an integrated HIV-1 long terminal repeat (LTR)–chloramphenicol acetyl transferase (CAT) reporter with Tat in the presence or absence of chloroquine. Chloroquine has been shown to prevent proteolysis of Tat in HeLa cells (*3*). Tat concentrations similar to those that inhibited lymphocyte proliferation caused transactivation in the H9 and U937 cells, but in contrast to the effect of chloroquine in HeLa cells, chloroquine had little effect (Fig. 2). The chloroquine-independent entry into lymphocytes and monocytes may reflect differences in cellular metabolism or mechanism of uptake.

Inhibition of antigen-induced proliferation of lymphocytes by Tat represents a novel activity of the protein. However, the mechanism of inhibition is unclear. Proliferation of blood mononuclear cells in response to soluble antigens involves multiple steps that include antigen processing and presentation, T cell activation, and lymphokine secretion (*9*). The inhibitory effect of

Table 1. Effect of HIV-1 Tat protein on antigen- and mitogen-induced lymphocyte proliferation. PBMCs were isolated by centrifugation over Ficoll-Hypaque. Cells (1×10^5) were incubated in microtiter plate wells with or without Tat (1.25 μg/ml) for 1 hour at 37°C in an atmosphere of 5% humidified CO_2 in 0.2 ml of RPMI 1640 medium containing 10% autologous serum, 5×10^{-5} *M* β-mercaptoethanol, glutamine, penicillin, and streptomycin. Soluble tetanus toxoid (0.1 − 0.5 μg/ml; Lederle Laboratories) or Candida antigen (1:30 dilution of *Candida albicans* allergenic extract; Greer Laboratories) was then added to the cultures. On day 6, cultures were pulsed for 4 hours with 1 μCi of [^{3}H]thymidine. Cells were harvested, and [^{3}H]thymidine incorporation into DNA was measured. For mitogen stimulation, 5×10^4 cells in 0.2 ml of RPMI containing 10% fetal bovine serum were incubated with PHA (Difco Laboratories) for 3 days. Data are mean counts per minute (cpm) ± 1 SD of six replicates. Numbers in parentheses indicate percent inhibition.

Stimulus	[^{3}H]Thymidine incorporation		
	Subject 1	Subject 2	Subject 3
Tetanus	19,874 ± 4,079	10,916 ± 1,678	61,789 ± 3,085
Tetanus + Tat	626 ± 106 (97%)	3,260 ± 1,125 (70%)	21,309 ± 1,767 (66%)
Candida	19,791 ± 6,304	2,856 ± 1,355	
Candida + Tat	1,695 ± 166 (91%)	702 ± 146 (75%)	
PHA	9,793 ± 516	7,022 ± 242	20,056 ± 2,312
PHA + Tat	13,125 ± 728 (0%)	6,454 ± 432 (8%)	17,835 ± 2,020 (11%)
Cells alone			
3-day culture	224 ± 67	436 ± 86	698 ± 125
6-day culture	765 ± 232	581 ± 190	429 ± 82
Cells + Tat			
3-day culture	221 ± 54	443 ± 164	392 ± 145
6-day culture	960 ± 163	1,188 ± 150	1,171 ± 357

Table 2. Specificity of immunosuppressive effect of Tat. Tat (1.25 μg/ml), oxidized Tat (1.25 μg/ml), or Tat (1.25 μg/ml) treated with rabbit polyclonal anti-Tat or preimmune rabbit serum, was incubated with 1×10^5 PBMCs for 1 hour before addition of tetanus toxoid (TT). Control cultures received tetanus but no Tat. On day 6, [^{3}H]thymidine incorporation was measured. To oxidize Tat, we bubbled 100% O_2 through a protein sample in 10 m*M* sodium phosphate buffer, *p*H 7.2, and 150 m*M* NaCl (PBS) for 1 hour at room temperature. Polyclonal anti-Tat was prepared by injecting rabbits with purified Tat (residues 1 to 72; 100 μg) three times, 2 weeks apart. To measure the effect of rabbit sera, we incubated 0.25 μg of Tat with 50% serum in 20 μl of PBS for 2 hours at 37°C before addition to PBMCs. Synthetic Tat 1–58 peptide was synthesized and purified as described (*8*). Data for oxidation are from one subject and are mean cpm ± 1 SD of quadruplicate replicates. Data for incubation with anti-Tat are from a different subject and are mean cpm ± 1 SD of quintuplicate replicates. Data for activity of the synthetic peptide are from a third subject and are mean cpm ± 1 SD of triplicate replicates. Experiments with other subjects gave similar results. Numbers in parentheses indicate percent inhibition.

Procedure	[^{3}H]Thymidine incorporation
Experiment 1	
TT	91,849 ± 17,035
TT + Tat (1.25 μg/ml)	57,503 ± 14,855 (37%)
TT + Tat oxidized (1.25 μg/ml)	82,940 ± 18,639 (10%)
Experiment 2	
TT	19,920 ± 6,004
TT + Tat (1.25 μg/ml)	1,934 ± 1,223 (90%)
TT + Tat (1.25 μg/ml) + anti-Tat serum	10,766 ± 3,299 (46%)
TT + Tat (1.25 μg/ml) + preimmune serum	3,020 ± 1,667 (85%)
Experiment 3	
TT	18,447 ± 2,577
TT + Tat (2.0 μg/ml)	8,819 ± 528 (52%)
TT + synthetic Tat 1–58 (20 μg/ml)	9,729 ± 1,984 (47%)

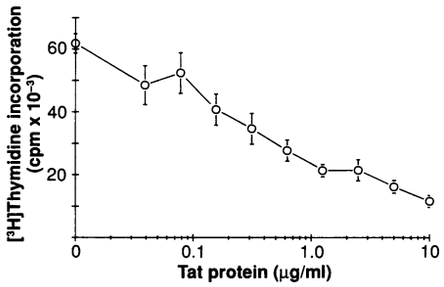

Fig. 1. Effect of HIV-1 Tat protein on tetanus-induced lymphocyte proliferation. Several concentrations of Tat were incubated with 1×10^5 PBMCs for 1 hour. Tetanus toxoid was then added to the cultures, and cells were grown as described in Table 1. Incorporation of [^{3}H]thymidine was measured on day 6. Results are means ± 1 SD of six replicates.

Tat could occur at any of these steps. Since Tat does not inhibit PHA-induced proliferative responses, it seems reasonable that Tat may interfere with signal transduction via the T cell antigen receptor or with antigen processing or presentation. Alternatively, Tat might induce production of cellular factors or cytokines that mediate the inhibition.

One of the hallmarks of acquired immunodeficiency syndrome (AIDS) is depletion of T4 cells, with the subsequent development of immunodeficiency. However, it is recognized that destruction of CD4$^+$ cells does not adequately explain all of the immunopathogenic effects of HIV infection (*10*). For example, even early in infection, patients' lymphocytes have a defect in their ability to recognize and respond to soluble antigen in vitro, although there are still normal numbers of CD4$^+$ T lymphocytes (*11*). In contrast, lymphocytes from HIV-1–infected patients retain the ability to proliferate in response to mitogens. The effect of Tat on in vitro lymphocyte proliferation mimicked this difference between antigen- and mitogen-induced proliferation. Under our in vitro conditions, 50 n*M* Tat was sufficient for 50% inhibition, suggesting that Tat may be a potent immunosuppressive agent. We do not yet know whether Tat must be provided extracellularly or whether Tat produced internally can elicit these effects. If inhibition of lymphocyte function occurs in vivo, Tat produced inside HIV-infected cells may act directly on those cells or Tat may be released extracellularly, perhaps by cell lysis. More studies are needed to determine the biological significance of an immunosuppressive activity of Tat.

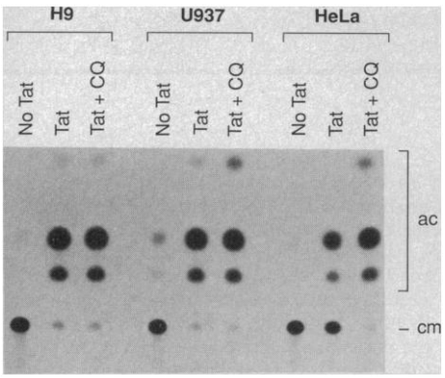

Fig. 2. Transactivation of the HIV-1 promoter in lymphocytes, promonocytes, and HeLa cells by purified Tat protein. H9 lymphocytes and U937 promonocytes (10^6 cells) that have an integrated HIV-1 LTR-CAT plasmid [H938 and U38 cells, respectively (*12*)] were incubated in RPMI 1640 medium containing 10% fetal bovine serum (1 ml in 25-mm wells) at 37°C (no Tat), treated with 5 μg of purified Tat protein (Tat), or treated with 5 μg of Tat protein and 100 μM chloroquine (Tat + CQ). Cells were harvested 24 hours after Tat treatment and assayed for CAT activity (*13*). HeLa cells (10^6 cells) that have an integrated HIV-1 LTR-CAT plasmid (HL3T1) (*12*) were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (1 ml in 25-mm wells) and similarly treated with Tat protein, or with Tat and chloroquine, and assayed for CAT activity. Unacetylated (cm) and acetylated (ac) forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography.

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Mechanisms for Regulating Expression of Membrane Isoforms of FcγRIII (CD16)

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Granulocyte and natural killer (NK) cell Fc receptors for immunoglobulin G (CD16) differ in only a few amino acids, yet have phosphatidylinositol glycan (PIG) or polypeptide membrane anchors, respectively. Mutagenesis shows that anchoring is regulated by a serine residue near the PIG anchor attachment site in the extracellular domain. The NK cell isoform was not expressed on the surface of COS cells unless cotransfected with a subunit that was expressed in NK cells and that was identical to the γ subunit of the high affinity IgE Fc receptor (FcεRI). However, the CD16 sequence and not expression of the γ subunit is dominant in regulating PIG reanchoring.

FC RECEPTORS (FcRs) SPECIFIC FOR the Fc domain of immunoglobulin (Ig) play a vital role in the function of the immune system. These receptors belong to the Ig gene superfamily (*1*). The IgG Fc receptor type III (FcγRIII or CD16) is predominantly found on NK cells, granulocytes, and tissue macrophages and mediates low affinity binding to human IgG1 and IgG3. On granulocytes two alleles, NA1 and NA2, have been described for this receptor. CD16 is anchored to the membrane of granulocytes through a PIG moiety, but on NK cells and tissue macrophages it has a peptide transmembrane anchor (*2–10*). The type of membrane anchor appears to determine the functional capacity of this receptor, because NK cell CD16 is able to trigger killing and other functions like lymphokine secretion and IL2 receptor expression, whereas granulocyte CD16 is unable to do so (*6, 11*). There are two similar genes for

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