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Human Immunodeficiency Virus Tat Induces Functional Unresponsiveness in T Cells

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Soluble proteins of the human immunodeficiency virus (HIV) might play a significant role in the pathogenesis of HIV infection. The effects of regulatory proteins of HIV, Tat, Nef, and Vif, on normal T-cell function were investigated. The addition of synthetic Tat peptides, but not that of the recombinant Nef or Vif protein, inhibited proliferative responses of CD4$^+$ tetanus antigen-specific, exogenous interleukin-2 (IL-2)-independent T-cell clones in a dose-dependent manner. In addition, Tat peptides inhibited the anti-CD3 monoclonal antibody-induced proliferative responses of both purified CD4$^+$ and CD8$^+$ T cells. Tat did not affect proliferative responses induced by phorbol myristate acetate plus ionomycin. The Tat peptides at the concentrations used (0.1 to 3 μg/ml) did not affect the viability of the cells as determined by trypan blue exclusion. Treatment of Tat peptides with polyclonal Tat antibodies abrogated the inhibitory effect of Tat. Soluble Tat proteins secreted by HeLa cells transfected with the *tat* gene also inhibited antigen-induced proliferation of the T-cell clones. Tat inhibited the anti-CD3 monoclonal antibody-induced IL-2 mRNA expression and IL-2 secretion but did not affect IL-2 receptor α-chain mRNA or protein expression on peripheral blood T cells. Finally, treatment of T-cell clones with the Tat peptide did not affect the antigen-induced increase in intracellular calcium, hydrolysis of phosphatidyl inositol to inositol trisphosphate, or translocation of protein kinase C from the cytosol to the membrane. These studies demonstrate that the mechanism of the Tat-mediated inhibition of T-cell functions involves a phospholipase C γ1-independent pathway.

The consequence of infection with human immunodeficiency virus type 1 (HIV-1) is a remarkable loss of cell-mediated functions. A major mechanism of immune suppression in AIDS is the depletion of circulating CD4$^+$ T cells, which play a central role in the regulation of immune responses (2, 33, 43). A functional abnormality of both CD4$^+$ and CD8$^+$ T cells in vivo in the early stages of HIV-1 infection and prior to the depletion of CD4$^+$ T-cell numbers has been the inability of peripheral blood lymphocytes (PBL) of human immunodeficiency virus (HIV)-infected individuals to generate antigen-specific T-cell-mediated responses (as measured by proliferation, interleukin-2 [IL-2] secretion, and cytotoxic T-cell responses to recall antigens, e.g., tetanus, influenza, and candida), with responses to alloantigens and to T-cell mitogens being unaffected (15, 22, 29). Several mechanisms for the specific defect observed have been postulated (12, 28). We and others have advocated the hypothesis that soluble proteins of HIV contribute to the qualitative defects in the early stages of HIV infection (8, 12, 31). In this respect, such envelope glycoproteins of HIV as gp120 inhibit antigen-specific T-cell proliferative responses in vitro by inhibiting the T-cell-receptor (TCR)-induced phospholipase C γ1 (PLC γ1) pathway (4, 5). T cells transfected with the HIV *nef* gene have been shown to downmodulate CD4 molecules (1) and repress IL-2 gene transcription by inhibiting activation of AP-1 and NF-κB (30). Several investigators (35, 37, 41) have now demonstrated that T cells treated with soluble HIV transactivating protein (Tat) and *tat*-transfected T cells are inhibited in their ability to respond to TCR-induced stimulation in vitro, the precise mechanism of which is yet to be clearly elucidated.

The *tat* gene product is responsible for the burst of replication in cells that have been stimulated with a foreign antigen (7). The Tat protein exerts its effects on the transacting response sequences in the 54 long terminal repeat of the HIV-1 promoter (36). Tat strongly enhances the efficiency or processivity of elongation of RNA polymerase II and can also affect the RNA initiation rate (19, 20). Uptake of and specific interaction with several molecules, both extracellular, e.g., dipeptidyl aminopeptidase (37), and intracellular, e.g., TFIID (21), suggest that exogenous Tat is capable of inducing biological effects in various cell types. Soluble Tat has been demonstrated in supernatants of HIV-infected and *tat*-transfected cells (11, 39). Uptake of exogenous Tat by cells not infected with HIV has been shown to be localized in the cytoplasm and nucleus (11, 26). Accumulating evidence suggests that Tat may exert stimulatory effects on cellular functions, e.g., growth of Kaposi's sarcoma cells (10) and upregulation of inflammatory cytokines IL-1, IL-6, tumor necrosis factor alpha (TNF-α) (3), and transforming growth factor beta (25). Tat has also been shown to repress transcription of several genes including major histocompatibility complex class I (16) and superoxide dismutase (13). Expression of *tat* mRNA in the lymph nodes and PBL of HIV-infected individuals (32) suggests a potential role of Tat in the immune suppression in vivo, especially during the acute phase of the disease.

Stimulation of the TCR initiates a cascade of biochemical events involving the activation of PLCγ1, which results in the hydrolysis of phosphatidylinositol-4,5-biphosphate which in turn yields the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (for a review, see reference 42). These second messengers are respectively responsible for the TCR-induced rapid and sustained increase in the concentration of

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intracellular calcium ions ($[Ca^{2+}]_i$) and the activation of protein kinase C (PKC). The increases in $[Ca^{2+}]_i$ and PKC activity have been shown to be required for the activation of transcription factors for the IL-2 gene promoter, which culminates in IL-2 secretion and effector functions. In this study, we have investigated the mechanism of HIV Tat-mediated inhibitory effects and demonstrated that the inhibition of IL-2 secretion by pretreatment of T cells with exogenous Tat does not involve the PLC γ1 pathway.

MATERIALS AND METHODS

Retroviral proteins. Full-length synthetic Tat peptides (amino acids 1 to 86) were prepared as described previously (18). Peptides were assembled on a phenylacetamidomethyl resin on an automated synthesizer. Peptides generated were purified by high-pressure liquid chromatography on a C8 column with acetonitrile-water gradients containing 0.1% trifluoroacetic acid. The amino acid composition of the peptides was determined by the Pico-Tag method and corresponded to the sequences of the HIV Tat protein of the HXB3 strain. For use, Tat was dissolved in RPMI medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethane-sulfonic acid) and degassed with nitrogen. Synthetic Tat-mediated transcription of the HIV long terminal repeat was determined in a cell-free transcription system (18). An *Escherichia coli*-derived recombinant Tat protein preparation was also purchased from Intracel, Cambridge, Mass. Recombinant Nef and Vif proteins were a gift from Carl Saxinger (National Cancer Institute, Bethesda, Md.) (6). Recombinant Rev protein was obtained from the National Institutes of Health AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, from David Rekosk, Marie-Louise Hammarskjold, and Michael Orsini.

Cells. PBL and lymphocyte subpopulations from healthy volunteers were isolated by Ficoll-Hypaque density gradient centrifugation as described previously (5). Purified T cells were isolated by rosetting twice with neuraminidase-treated sheep erythrocytes. The purity of the T cells was >98% as determined by anti-CD3 monoclonal antibody (MAb) staining (T3-FITC; Coulter Immunology, Hialeah, Fla.) analyzed by flow cytometry (EPICS ELITE; Coulter Electronics, Hialeah, Fla.). The nonrosetting fraction (non-T cells) was used as antigen-presenting cells (APC). $CD4^+$ and $CD8^+$ T cells were purified by negative selection utilizing magnetic beads (Dynal, Great Neck, N.Y.) according to the manufacturers' protocol. T cells were stimulated with anti-CD3 MAb (MAb 454, immunoglobulin G2a, a gift from Nicholas Chiorazzi, North Shore University Hospital, Manhasset, N.Y.) in the absence or presence of autologous irradiated non-T cells, and proliferation was measured by ^{14}C-thymidine incorporation as described previously (5).

$CD4^+$ tetanus, $CD45RO^+$ tetanus, and purified protein derivative antigen-specific T-cell clones, Tt 2.1, Tt 4.2, and PPD 3.5, were generated as described previously (5). Irradiated autologous Epstein-Barr virus-transformed B cells were used as APC. The clones were independent of exogenous IL-2 and secreted multiple cytokines upon stimulation with the appropriate antigen and APC.

HeLa cells cells were purchased from the American Type Culture Collection, Rockville, Md.; HeLa cells transfected with *tat* (HeLa-*tat* III) were obtained from the National Institutes of Health AIDS Repository (as a gift from W. Haseltine and E. Terwillinger) (39). In some experiments, polyclonal rabbit anti-Tat antibodies (Intracel) were used at various dilutions.

Signal transduction assays. (i) Measurement of inositol phosphate accumulation. Hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate was determined as described earlier (5). Briefly, T cells were labelled with 20 μCi of *myo*-2-[^{3}H]inositol (14 Ci/mM; New England Nuclear, Boston, Mass.) for 16 h, washed, and resuspended in a buffer containing 10 mM lithium chloride. After 30 min, the T cells were preincubated with medium, various concentrations of Tat, or gp120 for 2 h at 37°C. Reactions were initiated by the addition of tetanus antigen-pulsed APC and terminated after 10 min by the addition of 0.2 N HCl. Inositol metabolites were extracted with chloroform-methanol (1/2, vol/vol). Aqueous phases, removed by addition of chloroform and water, were neutralized and applied to an anion exchange column (Poly Prep AP 1× 8, 200/400 mesh formate form; Bio-Rad, Richmond, Calif.). After the removal of free inositol and glycerophosphoinositide by elution with water and 60 mM sodium formate–5 mM sodium tetraborate, IP1 was eluted with 0.2 M ammonium formate–0.1 M formic acid, IP2 was eluted with 0.4 M ammonium formate–0.1 M formic acid, and inositol-1,4,5-trisphosphate was eluted with 0.8 M ammonium formate–0.1 M formic acid. Radioactivity was assessed by liquid scintillation counting.

(ii) Measurement of protein kinase translocation. [^{3}H]phorbol 12-myristate 13-acetate ([^{3}H]PMA) (12.7 Ci/mM; New England Nuclear) binding assays were performed as described earlier (5). Briefly, T cells preincubated with medium, Tat, or gp120 were stimulated with tetanus antigen-pulsed APC. T cells, separated from Epstein-Barr virus-transformed B cells by Ficoll-Hypaque density gradient centrifugation, were lysed in a lysis buffer containing 0.5% Triton X-100 for 60 min and centrifuged at 100,000 × *g* for 20 min to separate membrane and cytosolic fractions. Assays were performed by making the following additions: 50 μl of the sample (membrane or cytosol fraction) in lysis buffer, 50 μl of phosphatidylserine (200 μg/ml), 50 μl of 12 nM ^{3}H-PMA in a binding buffer (50 mM Tris HCl [pH 7.4], 22.5 mM magnesium acetate, 1.5 mg of bovine serum albumin per ml), and 10 μl of 16 nM $CaCl_2$. Samples were transferred to a 37°C water bath for 15 min and returned to ice for 5 min. A 150-μl portion of polyethylene glycol 6000 (33.6% in 50 mM Tris HCl) was added to the sample, and the mixture was immediately vortexed. Samples were kept on ice for 10 min before centrifugation at 15,000 rpm for 15 min in a Microfuge. The supernatant was removed, and the pellet was washed once with phosphate-buffered saline (PBS) at 4°C. The radioactivity in the pellet (obtained by cutting the bottom of the tube) was counted in a liquid scintillation counter. Results were expressed as a percentage of counts translocated to the membrane from the cytosol (100 × disintegrations per minute in the membrane/disintegrations per minute in the cytosol).

(iii) Measurement of intracellular calcium. T cells were loaded with 10 μM Fluo 3AM for 1 h in PBS at room temperature. The fluorescence of the Fluo-3AM-loaded cells was monitored on an EPICS C flow cytometer as described previously (5) (excitation, 506 nm; emission, 526 nm). Maximal fluorescence was obtained by the addition of 4 mM $CaCl_2$, and minimum fluorescence was obtained by the addition of 2 mM $MnCl_2$. $[Ca^{2+}]_i$ was calculated as $0.45\ \mu M \cdot (F - F_{min})/(F_{max} - F)$, where F fluorescence, F_{min} is minimum fluorescence, and F_{max} is maximal fluorescence.

Reverse Transcriptase-PCR. Cells were stimulated with appropriate stimuli for 6 h. Total RNA was extracted as described earlier, and expression of mRNAs for IL-2, the IL-2 receptor α-chain, and actin was measured by reverse transcriptase PCR (40). Amplification of actin mRNA was used as a control for the amount of cDNA loaded.

Lymphokine assays. IL-2 secreted by the T-cell clones was assessed by proliferation of the IL-2-dependent CTLL-2 cell line as described previously (5).

RESULTS

Tat inhibits TCR-CD3-mediated proliferative responses of T cells. Figure 1A shows that the synthetic Tat peptide inhibits the antigen-induced proliferative responses of $CD4^+$ antigen-specific T-cell clones in a dose-dependent manner, with 50% inhibition at 0.1 μg/ml and 98% inhibition at 1 μg/ml. T-cell clones pretreated with various concentrations of other HIV-1 regulatory proteins added exogenously, recombinant Nef and Vif, were unaffected. Tat peptides did not inhibit stimulation of T-cell clones with PMA plus ionomycin. The inhibitory effect of Tat was confirmed with recombinant Tat proteins obtained commercially. The Tat peptides did not affect cell viability, as determined by trypan blue exclusion. As has been previously demonstrated (41), oxidation of the Tat peptides by bubbling oxygen abrogated their inhibitory effects on T-cell functions (data not shown).

The inhibitory effects of Tat could also be observed in peripheral blood T cells upon stimulation with anti-CD3 MAb. Figure 1B shows that pretreatment of purified T cells with Tat inhibited immobilized anti-CD3 MAb-induced proliferation; pretreatment with Nef, Vif, or Rev protein did not affect proliferation. In fact, Tat inhibited the anti-CD3 MAb-induced proliferative responses of both $CD4^+$ and $CD8^+$ T cells (Table 1). However, a higher concentration (3 μg/ml) of Tat was required to induce an 80% inhibition of T-cell function in peripheral blood T cells. Here again, viability of the cells was not affected by Tat, and pretreatment of these cells with Tat had no effect on proliferation induced by PMA plus ionomycin. The inhibitory effects of Tat could be abrogated by pretreatment with polyclonal anti-Tat antibodies, prior to the treatment of the T-cell clones, but not with normal rabbit serum (Fig. 2). Anti-Tat antibodies also inhibited the inhibitory effects of Tat on anti-CD3-induced proliferation of purified T cells (data not shown).

Tat inhibits antigen-induced IL-2 mRNA expression and IL-2 secretion but does not affect IL-2 receptor α-chain expression. Figure 3A shows that stimulation of T-cell clones with antigen and APC resulted in IL-2 secretion; pretreatment of T-cell clones with Tat resulted in inhibition of IL-2 secretion in a dose-dependent manner. IL-2 mRNA expression was induced in purified peripheral blood T cells upon stimulation

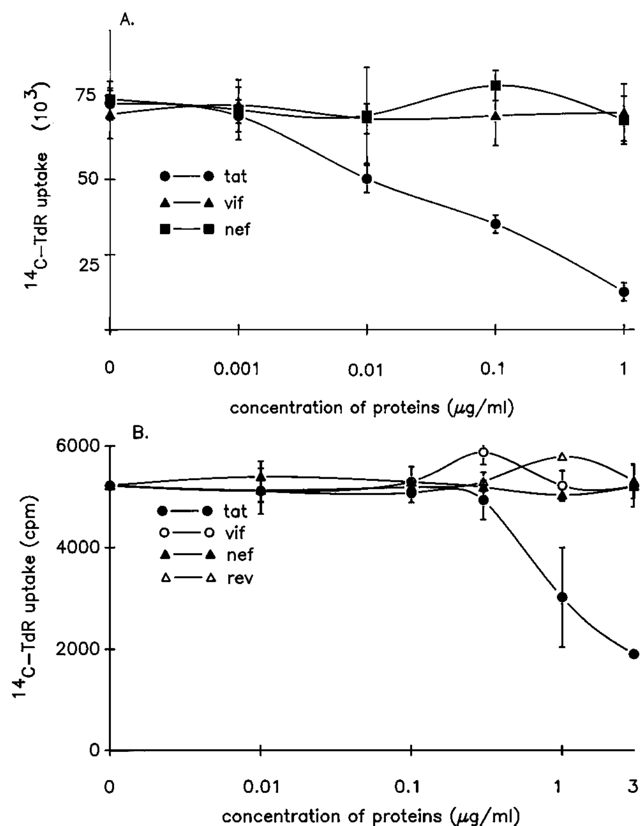

FIG. 1. (A) Tat inhibits antigen-specific proliferation of T-cell clones. T-cell clones (Tt 4.2) were pretreated with various concentrations of Tat, Vif, or Nef protein for 2 h at 37°C. Cells were then stimulated with tetanus antigen in the presence of irradiated autologous Epstein-Barr virus-transformed B cells (APC) for 3 days. Proliferation was measured by ^{14}C-thymidine incorporation. The results are representative of three separate experiments with two different clones. The vertical lines are standard deviations. (B) Tat inhibits immobilized anti-CD3 MAb-induced proliferation of peripheral blood T cells. T cells were pretreated with various concentrations of Tat, Vif, Nef, or Rev proteins for 2 h at 37°C. Cells were then cultured for 3 days in 96-well microtiter plates coated with anti-CD3 MAb. Proliferation was measured by ^{14}C-thymidine incorporation. The results are representative of two separate experiments from different donors. The vertical lines are standard deviations.

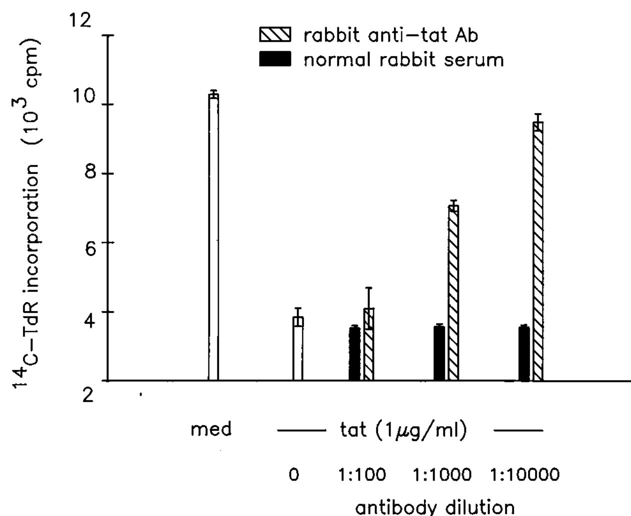

FIG. 2. Polyclonal anti-Tat antibodies abrogate the inhibitory effects of Tat on T-cell functions. Tat peptides were preincubated with various concentrations of rabbit anti-Tat antibodies or normal rabbit serum for 30 min at 4°C. T-cell clones were then pretreated with these proteins for 2 h at 37°C before stimulation with antigen and APC. Proliferation was measured by ^{14}C-thymidine incorporation. The results are representative of two separate experiments. The vertical lines are standard deviations.

with anti-CD3 MAb. Pretreatment of these cells with 3 μg of Tat per ml inhibited the ability of anti-CD3 MAb to induce IL-2 mRNA expression (Fig. 4). Treatment of T cells with Tat did not affect expression of mRNA from actin. The inhibitory effect of Tat on induction on IL-2 mRNA was abrogated by the addition of anti-Tat antibodies (data not shown). The anti-CD3 MAb-induced IL-2 receptor α-chain mRNA (Fig. 4) or protein expression (Fig. 3B) was not unaffected by pretreatment of T cells with Tat. These results indicate that Tat may induce T-cell unresponsiveness by inhibiting TCR-induced IL-2 gene transcription.

Addition of exogenous IL-2, but not of IL-1, IL-6, or TNF-α, overcomes Tat-induced inhibitory effects on T-cell proliferation. T-cell clones were pretreated with 1 μg of Tat per ml for 2 h at 37°C and stimulated with antigen and APC. The addition of increasing concentrations of recombinant IL-2 overcame the inhibitory effect of Tat on T-cell proliferation (Fig. 5); addition of a combination of IL-1, IL-6, and TNF-α, however, failed to overcome the inhibitory effect of Tat on T-cell responses. These observations suggest that Tat might suppress TCR-induced proliferative responses mainly by inhibiting IL-2 secretion by T cells.

Tat-induced inhibitory effects do not affect TCR-mediated signal transduction events following the PLC γ1 pathway.

TABLE 1. Tat inhibits proliferative responses of peripheral blood T cells[a]

T cell	Pretreatment with Tat (μg/ml)	^{14}C-thymidine incorporation (cpm) after stimulation with[b]:		
		Medium	Anti-CD3 MAb	PMA with ionomycin
CD4$^+$	0	80 ± 23	5,760 ± 121	10,096 ± 1,643
	0.1	86 ± 17	5,896 ± 391 (0)	12,065 ± 809 (0)
	1.0	80 ± 12	4,061 ± 306 (29)	10,649 ± 409 (0)
	3.0	89 ± 23	783 ± 109 (86)	12,664 ± 1,218 (0)
CD8$^+$	0	100 ± 6	3,006 ± 751	6,587 ± 529
	0.1	106 ± 9	3,109 ± 267 (0)	6,400 ± 239 (1)
	1.0	108 ± 19	2,386 ± 402 (20)	6,560 ± 108 (0)
	3.0	127 ± 16	1,080 ± 149 (64)	6,309 ± 348 (4)

[a] CD4$^+$ T cells and CD8$^+$ T cells at concentrations of 10^6/ml were pretreated with various concentrations of Tat peptides for 2 h at 37°C and stimulated with anti-CD3 MAb or a combination of PMA plus ionomycin. CD4$^+$ and CD8$^+$ T cells were cultured in the presence of autologous, irradiated non-T cells as APC. The results are representative of two separate experiments.

[b] Numbers in parentheses are percent inhibition induced by pretreatment with Tat compared with that for untreated T cells. Values are means ± standard deviations.

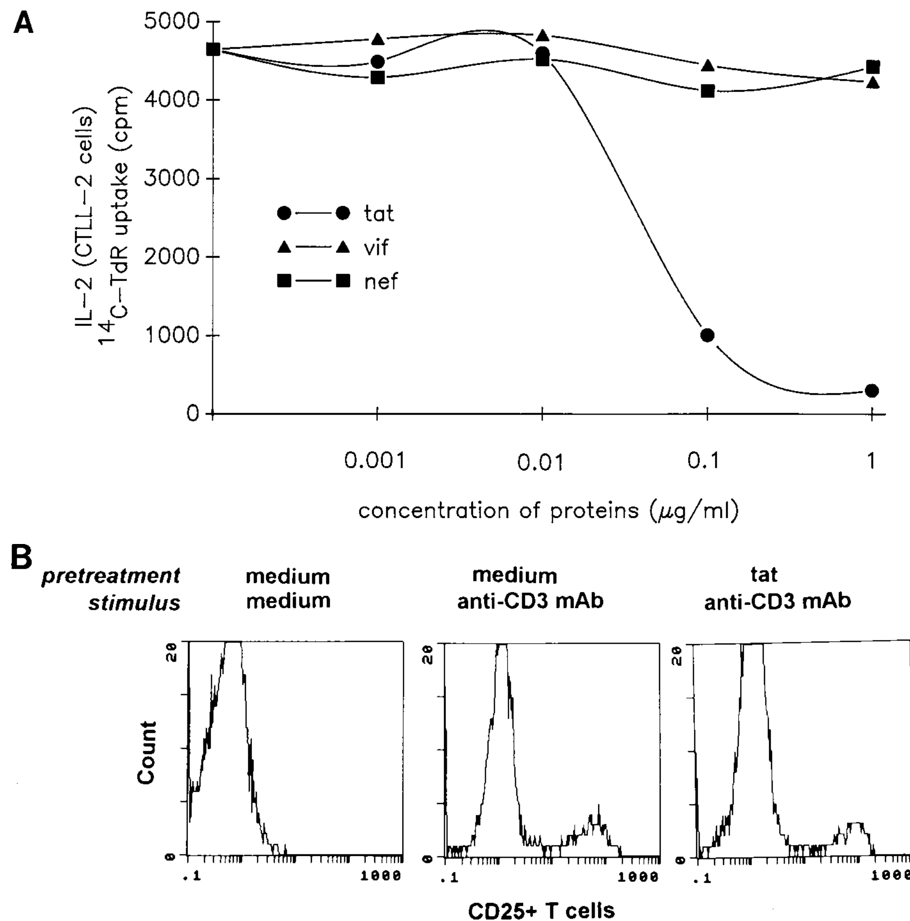

FIG. 3. (A) Tat inhibits TCR-induced IL-2 secretion. T-cell clones (Tt 4.2) were pretreated with various concentrations of Tat, Vif, or Nef protein for 2 h at 37°C. Cells were then stimulated with the tetanus antigen in the presence of irradiated autologous Epstein-Barr virus-transformed B cells (APC) for 24 h. IL-2 secretion was determined by the ability of the culture supernatants to induce proliferation of IL-2-dependent CTLL-2 cells. The results are representative of three separate experiments with two different clones. (B) Tat does not affect anti-CD3 MAb-induced IL-2 receptor α-chain expression. Purified T cells (either untreated or pretreated with Tat) were stimulated with anti-CD3 MAb in the presence of autologous irradiated APC for 48 h at 37°C. IL-2 receptor α-chain expression was analyzed by flow cytometry.

Purified protein derivative-specific T-cell clones were pretreated with medium, Tat, or gp120 and stimulated with purified protein derivative antigen-pulsed autologous APC for various time intervals as indicated in Fig. 6. Preincubation of T cells with Tat peptides failed to inhibit an antigen-induced increase in intracellular calcium, hydrolysis of phosphatidylinositol to inositol phosphates, and activation of PKC (Fig. 6A to C). As we have previously demonstrated (5), pretreatment of T-cell clones with envelope glycoproteins inhibited these signalling events.

Tat proteins secreted by HeLa cells transfected with the *tat* gene can inhibit T-cell responses. Cultures were established in dual-chamber plates (Transwell) with the HeLa-*tat* cell line, which secretes Tat proteins (39), or with control HeLa cells which were separated from T-cell clones plus APC by a 0.45-μm-pore-size membrane which allows only soluble proteins to pass through. Cultures of HeLa-*tat* cells, but not control HeLa cells, inhibited antigen-induced proliferation by antigen-specific T-cell clones in this system (Fig. 7, neat). The inhibitory effect of the HeLa-*tat* cell culture supernatant was further investigated by the addition of various dilutions of the culture supernatants to antigen-stimulated T-cell cultures and was found to be dose dependent with regard to T-cell functions. The specificity of the inhibitory effects was confirmed by

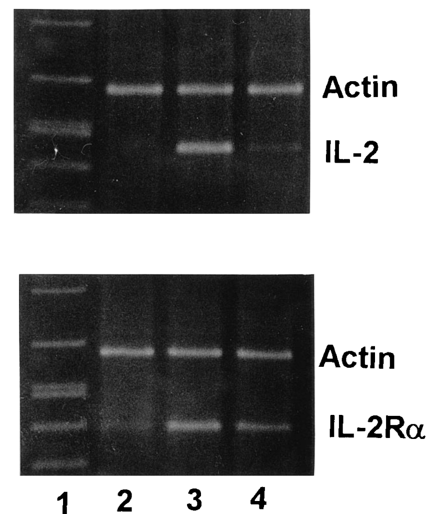

FIG. 4. Tat inhibits anti-CD3 MAb-induced IL-2 mRNA expression but does not affect IL-2 receptor α-chain expression. Purified T cells (either untreated [lanes 2 and 3] or pretreated with Tat [lane 4]) were stimulated with medium alone (lane 2) or anti-CD3 MAb (lanes 3 and 4) in the presence of autologous irradiated APC for 6 h at 37°C. The cells were harvested, the RNA was extracted, and the mRNAs for IL-2, the IL-2 receptor α-chain, and actin were determined by reverse transcriptase PCR. Lane 1 shows size markers. The results are representative of two experiments.

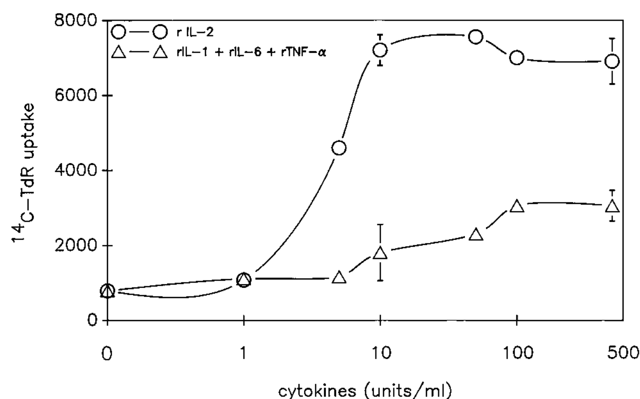

FIG. 5. Addition of exogenous IL-2, but not of IL-1, IL-6, or TNF-α, restores Tat-induced inhibitory effects on T-cell proliferation. T-cell clones were pretreated with Tat (1 μg/ml) for 2 h at 37°C and stimulated with antigen and APC in the presence of increasing concentrations of recombinant IL-2 or a combination of IL-1, IL-6, and TNF-α. Proliferative responses were measured by ^{14}C-thymidine incorporation. The vertical lines are standard deviations.

the ability of the anti-Tat antibody to abrogate the inhibitory effects of HeLa-*tat* supernatants on T-cell functions.

DISCUSSION

We have demonstrated that HIV Tat inhibits TCR-mediated IL-2 mRNA expression, IL-2 secretion, and the proliferative responses of both CD4$^+$ and CD8$^+$ T cells by mechanisms independent of the PLC γ1 pathway; induction of IL-2 receptor α-chain expression, however, remains unaffected.

Depression of antigen-specific T-cell responses, a relatively early feature of HIV infection, involves a qualitative dysfunction of both CD4$^+$ and CD8$^+$ T cells (15, 29, 34). The mechanism of this defect in T cells has been attributed in part to the inhibitory effects of proteins coded for by viral genes, e.g., *env* and *nef* (1, 4, 5, 30). Studies with exogenous Tat and *tat*-transfected T cells have suggested that this regulatory protein may also play a role in immune suppression in HIV-1 infection (35, 37, 41); the mechanism of action and its definitive role in vivo, however, remain obscure.

The PLC γ1 pathway has been shown to play a central role in the induction of IL-2 secretion following TCR stimulation (9). Delineations of distinct signalling pathways upon T-cell activation, e.g., those of the mitogen-activated protein kinase pathway, the cyclic AMP pathway, the phosphatidylinositol-3 kinase pathway, and the ceramide pathway, have recently demonstrated requirements for several complex intermolecular interactions involving phosphorylation and dephosphorylation events which culminate in IL-2 secretion (42). Signalling events immediately downstream of the antigen-TCR ligation indeed utilize the PLC γ1 activation, resulting in second messengers, inositol-1,4,5-trisphosphate and diacylglycerol (42). In this study, pretreatment of T cells with Tat did not affect the TCR-induced increase in the $[Ca^{2+}]_i$, hydrolysis of phosphatidylinositol-4,5-bisphosphate, and activation of PKC, although IL-2 secretion was markedly reduced. These observations suggest that Tat may affect TCR-induced IL-2 secretion by two possible mechanisms: (i) by affecting signals downstream of phosphatidylinositol-4,5-bisphosphate hydrolysis, increasing the $[Ca^{2+}{}_i]$, and activating PKC or (ii) by affecting another pathway critical for IL-2 secretion (e.g., the mitogen-activated protein kinase, phosphatidylinositol-3 kinase, or cyclic AMP pathway).

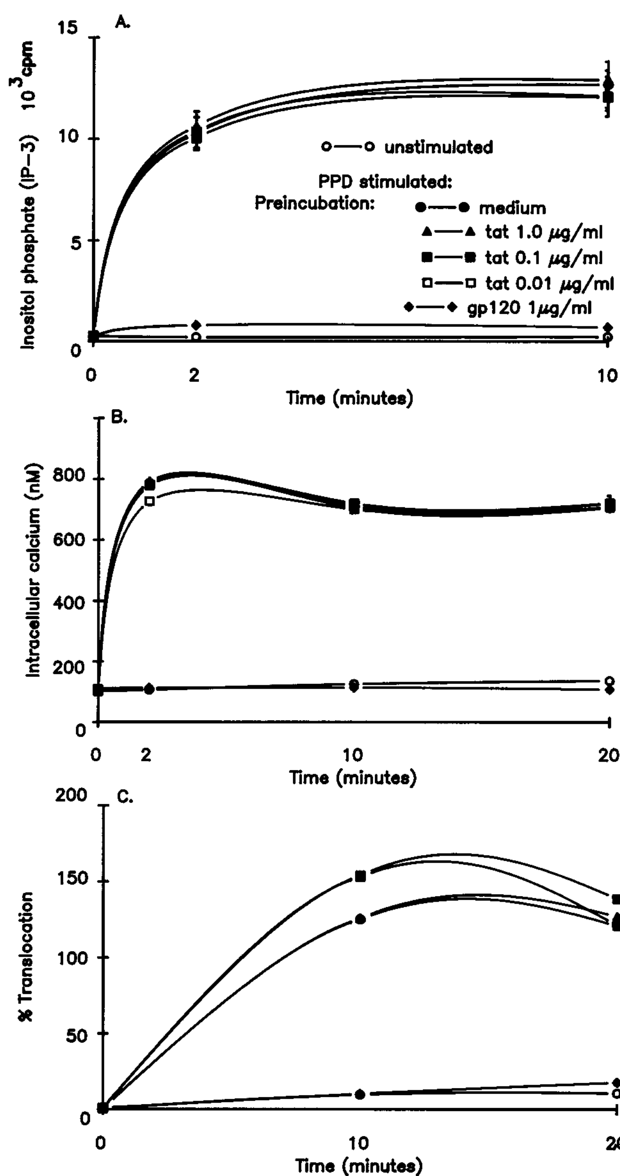

FIG. 6. Tat-induced inhibitory effects do not affect TCR-mediated signal transduction events (hydrolysis of phosphatidylinositol [A], increase in intracellular calcium [B], and activation of PKC [C]). T-cell clones were pretreated with Tat or gp120 and stimulated with antigen-pulsed APC for various time intervals as indicated. Assays were performed as described in Materials and Methods. The vertical lines are standard deviations.

Stimulation of T cells with a combination of PMA plus ionomycin has been suggested to bypass the requirement for receptor-ligand interaction and to induce IL-2 secretion by activating the signal transduction cascade downstream of the TCR-induced PKC activation and increase in the $[Ca^{2+}]_i$. Since treatment of T-cell clones with Tat did not affect proliferative responses induced by PMA plus ionomycin, it is possible that Tat exerts its inhibitory effects by influencing another pathway required for IL-2 secretion. In this respect, it has been previously demonstrated that Tat binds to and inactivates the enzymatic activity of dipeptidyl aminopeptidase (CD26) (37). The role of dipeptidyl aminopeptidase in the regulation of IL-2 secretion, however, is still unclear. In our culture system, the addition of exogenous Tat failed to downmodulate CD26 expression on T cells. The contradictory

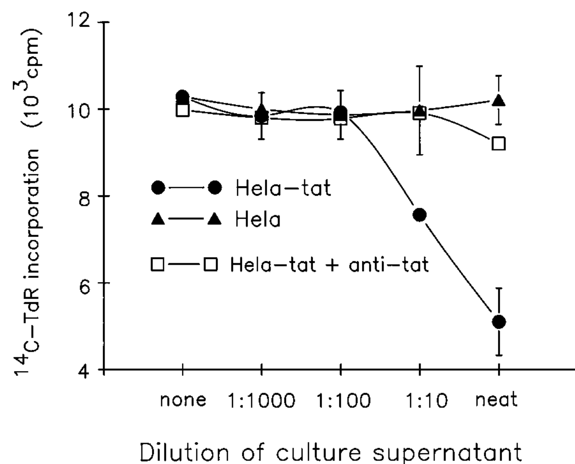

FIG. 7. Tat proteins secreted by HeLa cells transfected with the *tat* gene can inhibit T-cell responses. The HeLa-*tat* cell line or control HeLa cells were separated from T-cell clones plus APC by culturing them in dual-chamber plates separated by a 0.45-μm-pore-size membrane (neat). The ability of various dilutions of these supernatants to inhibit responses was determined by pretreating the T-cell clones. The specificity of Tat-induced inhibition was analyzed by the addition of a 1:100 dilution of anti-Tat antibodies (anti-Tat). Antigen- and APC-induced proliferation was measured by ^{14}C-thymidine incorporation. The vertical lines are standard deviations.

results may be due to the different cell cultures and MAbs used. Preliminary results have indicated that Tat transiently downmodulates CD45 expression on T cells (4a). Studies are currently in progress to determine whether Tat affects CD45-associated phosphatase activity, which plays an important role in TCR-mediated signal transduction (23).

Induction of proliferative responses in T cells requires the secreted IL-2 to bind to the IL-2 receptor. The IL-2 receptor has been shown to comprise three polypeptide chains, the α-chain, β-chain, and γ-chain (38). Although IL-2 secretion was inhibited, pretreatment of T cells with Tat did not affect the expression of the IL-2 receptor α-chain at either the mRNA or protein level. These observations suggest that two distinct signals are transduced through the TCR complex: one signal is involved in IL-2 gene transcription and is inhibited by Tat, and another, which activates the IL-2 receptor α-chain gene, is unaffected by Tat.

The supposed inhibitory effect of exogenous Tat on T-cell function in vivo has recently been refuted because of the finding that Tat inhibits the proliferative response only of purified T cells and not of T cells in the presence of APC (27). It had been suggested that soluble factors secreted by APC could overcome the inhibitory effect of Tat on T-cell function. In our study, exogenous addition of recombinant IL-1, IL-6, and TNF-α (cytokines predominantly secreted by APC) failed to overcome the inhibitory effect of Tat on antigen-induced proliferative responses of T-cell clones; exogenous IL-2 (secreted by T cells), however, completely restored T-cell function.

Several investigators have now demonstrated that exogenous Tat exerts significant biological effects in vitro. The role of exogenous Tat in vivo, however, remains questionable. Recent studies (14, 32) have demonstrated that HIV replication is active in the lymphoid tissue and PBL throughout the course of the disease. Expression of *tat* mRNA in lymph nodes and mononuclear cells has been shown in the early (acute) phase of the disease. It is conceivable that Tat proteins secreted by HIV-infected cells may contribute to the qualitative defect in uninfected T cells in lymph nodes, especially in early disease states, and in PBL during the more aggressive late phases of the disease. In this study, the ability of supernatants of HeLa-*tat* cells to inhibit T-cell functional responses suggests that Tat secreted by HIV-infected cells in vivo may inhibit the functional responses of bystander T cells not infected with HIV. Furthermore, unlike gp120, Tat affects the functional responses of both CD4$^+$ as well as CD8$^+$ T cells, a phenomenon observed in vivo in HIV-infected individuals (15, 29, 34). Further studies to understand the mechanism of the inhibitory effects of Tat on T-cell function will provide new insights into the immunopathogenesis of AIDS, especially during the acute phase of the disease. These studies may also have important implications for the therapeutic trials of Tat antagonists for the control of HIV infection (17, 24).

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