Tat Protein of Human Immunodeficiency Virus Type 1 Induces Interleukin-10 in Human Peripheral Blood Monocytes: Implication of Protein Kinase C-Dependent Pathway

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The clinical manifestations observed in human immunodeficiency virus type 1 (HIV-1)-infected patients are primarily due to the capacity of the virus and its components to inactivate the immune system. HIV-1 Tat protein could participate in this immune system disorder. This protein is secreted by infected cells of HIV-infected patients and is free in the plasma, where it can interact and be taken up by both infected and non-infected cells. In asymptomatic patients infected by HIV-1, production of interleukin-10 (IL-10), a highly immunosuppressive cytokine, is associated with disease progression to AIDS. In the present work, we tested the capacity of Tat to induce IL-10 production by peripheral blood monocytes of healthy donors. The results show that Tat causes the production of IL-10 in a dose- and stimulation time-dependent manner. Investigations of the mechanisms involved in signal transduction show that (i) the calcium pathway is not or only slightly involved in Tat-induced IL-10 production, (ii) the protein kinase C pathway plays an essential role, and (iii) monocyte stimulation by Tat results in the intranuclear translocation of transcription factor NF-kB and in the induction of phosphorylation of the mitogen-activated protein kinases ERK1 and ERK2; activation of these two potential substrates of protein kinase C is required for the production of IL-10. Finally, our results suggest that the effect of Tat is exerted at the membrane level and that the active domain is located within N-terminal residues 1 to 45. This production of IL-10 induced by Tat could participate in the progression of HIV infection to AIDS.

The clinical manifestations observed in patients infected by human immunodeficiency virus (HIV) are primarily due to the capacity of the virus to inactivate the immune system. Before the decrease in the number of CD4 T lymphocytes, a disorder in the immune response is observed (17, 18, 38). The cellular and molecular mechanisms of this deficiency of the immune system cannot be explained solely by the direct lytic effect of the virus on infected CD4 T lymphocytes. HIV-1 can infect target cells and remain in the form of a latent provirus. In addition to this mechanism to escape the defenses of the immune system, the virus uses other strategies involving viral and cellular factors.

One of the potential candidates is HIV Tat gene product, a 14-kDa protein known for its transactivating activity on the viral genome (34). Tat binds to the secondary-structure sequence TAR (Tat activation region) 5’ of viral RNA during transcription and thereby enables the recruitment of cellular factors forming the complex of cyclin T1 and cdk9, called TAK (Tat-associated kinase), that phosphorylates the C-terminal domain on RNA polymerase 2, thereby activating transcription elongation (28). Tat also participates in the pathogenesis of HIV-1 infection by its capacity to interact with different cell types. Tat is found in the serum of HIV-infected patients (26, 54). It is secreted by infected cells (26) and can act on other cells, whether or not they are infected (10, 21, 22, 39, 57). Tat activates quiescent CD4+ T cells, rendering them permissive for HIV-1 infection (37). This effect is accentuated by the capacity of Tat to increase the rate of expression of coreceptors for the chemokines CXCRL4 and CCR5 (29). Tat also contributes to immune system disorders by inducing apoptosis of T lymphocytes (36). Tat interferes with the cell-mediated immune response by inhibiting major histocompatibility complex class I molecule expression, as reported for Jurkat cells (30), NK cell activity (59), and interleukin-12 (IL-12) production by dendritic cells (45) and by monocytes (31). It is now established that deregulation of cytokine production contributes to the attenuated functioning of the immune system in the course of HIV-1 infection. HIV-1-infected patients thus develop a progressive decrease in the TH1-type cellular immune response that results in an increase in the TH2-type humoral immune response mediated by IL-4, IL-6, and IL-10 (16, 17, 38).

The infection of T-cell (H9) or promonocytic (U937) lines by HIV in vitro stimulates the secretion of IL-10 (40). In line with these reports, Shearer’s group, in a study including more than 1,000 patients (18), identified four patient classes depending on the capacity of their CD4 T lymphocytes to respond to different stimuli (mitogen, alloantigen, influenza virus, and HIV-1 antigens). The progressive loss of the response of the immune system to these stimuli was found to be associated with a course leading to AIDS. Considerable production of IL-10 by peripheral blood mononuclear cells (PBMC) was observed in these patients and paralleled the alteration in CD4+ T-cell proliferative function (18). In addition, the immunosuppressive effect of IL-10 also correlated with the capacity of isolated mononuclear cells of patients infected by HIV and immunodepressed to proliferate in vitro after stimulation by peptide antigens of the HIV envelope glycoproteins in the presence of a neutralizing anti-IL-10 antibody (18).

The production of cytokines involves primarily two signaling pathways, the calcium pathway and activation of protein kinase
C (PKC) (13, 3). The simultaneous use of a calcium ionophore such as ionomycin and a PKC activator such as phorbol myristate acetate (PMA) would thus lead to the stimulation of production of most cytokines (in particular IL-2 and IL-4). These pathways are activated following the binding of a ligand to its receptor. Activated phospholipase C (PLC) cleaves phosphatidylinositol biphosphate to inositol 1,4,5-triphosphate, responsible for the mobilization of intracellular calcium, and to diacylglycerol, which initiates PKC activation. These two pathways lead to the phosphorylation and activation of cellular proteins (mitogen-activated protein MAP kinases) and of transcription factors (NF-AT, NF-kB, AP-1, and CREB) responsible for the induction of cytokine genes (25).

The HIV-mediated production of IL-10, a cytokine with immunosuppressive properties (41), seems to be a crucial event during HIV infection.

The aims of the present work were to determine if Tat could have a direct effect on human monocytes, a prime target of HIV but also a key cell in the immune system, by inducing the production of IL-10 and to elucidate the intracellular mechanisms responsible for this production of IL-10.

Our results show that Tat from HIV-1 induces the production of IL-10 by human peripheral blood monocytes. This IL-10 production is highly dependent on the activation of PKC.

**MATERIALS AND METHODS**

**Monocyte isolation.** PBMC were isolated from blood or the buffy coat from healthy HIV-negative donors in a Ficoll density gradient (Pharmacia). The PBMC were resuspended in 60/30 complete medium (60% AIM V and 30% Iscove [Gibco]) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (FCS). PBMC were then plated at a density of 10⁶ cells/well in 24-well Primaria (Becton Dickinson) tissue culture plates. After 24 h of culture at 37°C in 5% CO₂, nonadherent cells were removed, and the remaining cells were washed twice and then incubated with the different compounds tested.

**Recombinant Tat protein.** (i) Native recombinant Tat. Recombinant HIV-1 Tat protein was obtained from Agence Nationale de la Recherche sur le SIDA (Paris, France). The level of endotoxin contamination in purified HIV-1 Tat was assessed by using the Limulus amebocyte lyase assay (BioSepra, Villefranche la Garenne, France). HIV-1 Tat protein contained less than 0.3 EU/μg. This Tat preparation has been shown to be biologically active (7, 53).

(ii) Chemicaly oxidized Tat. Native recombinant Tat was oxidized with 3% H₂O₂ in phosphate-buffered saline (PBS) for 1 h at 25°C as previously described (19). In contrast to unmodified native Tat, no transcription activity was found with this oxidized Tat (data not shown), in accordance with data reported by Cohen et al. (19).

(iii) Tat mutants. HIV Tat mutants were produced as glutathione-S-transferase (GST) fusion proteins in Escherichia coli. The wild-type GST-Tat 1-101 and Tat-deleted mutants GST-Tat 1-72, GST-Tat 1-55, GST-Tat 1-45, GST-Tat 20-72, and GST-Tat 30-72 were purified as previously described (8). As a control, GST was purified in the same conditions and used in the same experiments. All human recombinant polypeptide was free (less than 0.3 EU/μg) and biologically active, as previously described (8).

**Signal transduction experiments.** Isolated monocytes were cultured in 60/30 complete medium in the absence or presence of HIV-1 Tat protein or PBS. HIV-1 Tat (3.6 × 10⁴ M) and PBS (200 μg/ml) were prepared as stock solutions in PBS and water, respectively. Further dilutions were done in FCS-free medium.

Monocytes were incubated for 30 min with various signal transduction pathway inhibitors, and HIV-1 Tat (10 nM) was added for an additional 24 h. The following inhibitors were used: U73122 (1-[6-((17α)-3-fluo-acetoxymethylester (AM; Molecular Probes, Leiden, The Netherlands) for 30 min at 37°C. Intracellular Ca²⁺ concentrations were measured in cells stimulated by Tat (10 or 100 nM) or 1 μM ionomycin (Sigma). Ionomycin was initially dissolved in DMSO; after washing, plates were incubated with the substrate O-phendylendiamine dihydrochloride plus H₂O₂ (Sigma). The reaction was stopped by adding 50 μl of H₂SO₄ (4 N) to each well. Absorbance was read at 490 nm, with a wavelength correction of 600 nm. Cytoplasmic Ca²⁺ concentrations were determined from flow cytometric measurements of recombinant human anti-human IL-10 polyclonal antibody (BASF217), obtained from R & D Systems, for 2 h at room temperature. After washing, the bound biotinylated polyclonal antibody was visualized by an additional 20 min of incubation with streptavidin-peroxidase (Sigma, Saint Quentin Fallavier, France) diluted 1:4000 in 0.1 M PBS/0.1% BSA. After washing, plates were incubated with the substrate 4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) per ml in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride. X-Gal had been previously dissolved in DMSO at 40 mg/ml. The enzymatic reaction was stopped by removing the X-Gal reaction mixture. Stained cells were stored in PBS buffer.

**IL-10 detection by ELISA.** IL-10 production was quantified by using a two-site sandwich enzyme-linked immunosorbent assay (ELISA). MAB217 (R & D Systems, Oxon, U.K.) monoclonal antibody (MAB) (4 μg/ml) was used for capture overnight at room temperature. After three washes with PBS containing 0.05% Tween 20 (washed buffer), plates were blocked by adding 1% bovine serum albumin and 5% sucrose to each well for a minimum of 1 h. After three washes, culture supernatants (100 μl) were incubated for 2 h at room temperature. Plates were then washed three times and incubated with biotinylated anti-human IL-10 polyclonal antibody (BASF217), obtained from R & D Systems, for 2 h at room temperature. After washing, the bound biotinylated polyclonal antibody was visualized by an additional 20 min of incubation with streptavidin-peroxidase (Sigma, Saint Quentin Fallavier, France) diluted 1:4000 in 0.1 M PBS/0.1% BSA. After washing, plates were incubated with the substrate 4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) per ml in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM magnesium chloride. X-Gal had been previously dissolved in DMSO at 40 mg/ml. The enzymatic reaction was stopped by removing the X-Gal reaction mixture. Stained cells were stored in PBS buffer.

**Intracellular Ca²⁺ concentrations.** Intracellular Ca²⁺ concentrations were determined from fluorescence micrographs obtained with an inverted Diaphot microscope (Nikon) and observed with a 40× objective. The excitation wavelength was 490 nm, with a 525-nm barrier filter. Fluorescence was detected by an intensified charge coupled device camera (C2400-80; Hamamatsu, Photonics, Hamamatsu, Japan). With the magnification used (40×), a field of 200 by 200 μm was recorded by the camera. Three to five fields were observed for each type of experiment, and in each field 12 windows (9 × 9 μm) were distributed on different parts of the plate. Cells were incubated with 5 μM 3-fluo-acetoxymethylster (AM; Molecular Probes, Leiden, The Netherlands) for 30 min at 37°C. Intracellular Ca²⁺ concentrations were measured in cells stimulated by Tat (10 or 100 nM) or 1 μM ionomycin (Sigma). Ionomycin was initially dissolved in DMSO at a concentration of 2 mM. Cell preparations were placed on the stage of an inverted Diaphot microscope (Nikon) and observed with a 40× objective. The excitation wavelength was 490 nm, with a 525-nm barrier filter. Fluorescence was detected by an intensified charge coupled device camera (C2400-80; Hamamatsu, Photonics, Hamamatsu, Japan). With the magnification used (40×), a field of 200 by 200 μm was recorded by the camera. Three to five fields were observed for each type of experiment, and in each field 12 windows (9 μm) were distributed on different parts of the plate. Cells were incubated with 5 μM 3-fluo-acetoxymethylster (AM; Molecular Probes, Leiden, The Netherlands) for 30 min at 37°C. Intracellular Ca²⁺ concentrations were measured in cells stimulated by Tat (10 or 100 nM) or 1 μM ionomycin (Sigma). Ionomycin was initially dissolved in DMSO at a concentration of 2 mM. Cell preparations were placed on the stage of an inverted Diaphot microscope (Nikon) and observed with a 40× objective. The excitation wavelength was 490 nm, with a 525-nm barrier filter. Fluorescence was detected by an intensified charge coupled device camera (C2400-80; Hamamatsu, Photonics, Hamamatsu, Japan). With the magnification used (40×), a field of 200 by 200 μm was recorded by the camera. Three to five fields were observed for each type of experiment, and in each field 12 windows (9 μm) were distributed on different parts of the plate.

**EMSA.** For the electrophoretic mobility shift assay (EMSA), nuclear extracts were prepared as previously described (30). Briefly, cold Tris-buffered saline (TBS, pH 7.4) was added to monocytes (2 × 10⁶ cells), which were scraped and harvested after 16 h of incubation. Monocytes, whole PBMC, or monocyte-depleted PBMC (2 × 10⁶ cells) were washed and collected. Cells were transferred to 1.5-M Ependorf tubes and microcentrifuged at 4°C for 15 s. The pellet was washed twice in 400 μl TBS and re-centrifuged. Cells were then resuspended in 10 mM HEPES [pH 7.9], 0.1 mM EGTA, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After 15 min on ice, 25 μl of 10%
Nonidet P-40 (Sigma) solution was added to the samples, and cells were homogenized with a Vortex and microcentrifuged at 4°C for 30 s. The pellets were resuspended in 50 ml of lysis buffer (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), and the cells were agitated vigorously for 15 min at 4°C on a shaking platform. The nuclear extracts were microcentrifuged for 5 min at 4°C, and aliquots of supernatants were frozen at −80°C.

Total protein levels were determined with the Bradford assay, using a commercial protein assay reagent (Bio-Rad, Ivry Sur Seine Cedex, France). The NF-κB mobility shift assays were performed using 6 mg of protein of nuclear extract, 104 cpm of radiolabeled double-stranded NF-κB probe in C buffer (100 mM KCl, 1 mM DTT, 1 mM ZnSO4, 20% glycerol, 0.01% Nonidet P-40, 50 mM HEPES [pH 7.9]) supplemented with bovine serum albumin, tRNA, and poly(dI:dC) in a final volume of 20 μl. After 20 min at room temperature, electrophoresis of the mixture was carried out at 120 V in a 5% polyacrylamide gel.

Two oligonucleotide sequences were used. The first was the HIV-1 LTR NF-κB sequence 5'-GCTGGGGACTTTCCAGGGAG-3', and in order to determine specificity of binding, the second was the NF-κB mutated sequence 5'-GCTGTTTACTGGCCCAGGGAG-3'.

SDS-PAGE and Western blot. After incubating cells (10⁶) in the presence or absence of Tat or PMA for 15 or 30 min, cold TBS (pH 7.8) was added, and the cells were transferred to 1.5-ml Eppendorf tubes and microcentrifuged at 4°C for 15 s. The pellet was resuspended in 200 μl of lysis buffer (10 mM HEPES [pH 7.9], 0.1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF). After 15 min on ice, 7 μl of 10% Nonidet P-40 solution was added to the samples and the cells were homogenized with a Vortex. The cytoplasmic extracts were microcentrifuged at 4°C for 30 s, and the supernatants were stored at −80°C until used.

Generated extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were transferred to nitrocellulose membranes. Immunoblotting was conducted using either rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody (1:1,000) (New England Biolabs, Hertfordshire, England) or rabbit anti-p44/42 MAP kinase antibody (1:1,000) (New England Biolabs). Membranes were incubated with the primary antibody (2 h at room temperature). Immunoreactive bands were then detected by incubation for 2 h at room temperature with swine anti-rabbit immunoglobulins conjugated with horseradish peroxidase (1:1,000) (Dako A/S, Roskilde, Denmark). The membranes were then visualized using a chemiluminescent substrate (Pierce, Rockford, Ill.).

Statistical analysis. The Mann-Whitney nonparametric test was used in this study to compare data for stimulated cells in the absence and presence of inhibitors.

**RESULTS**

Tat induces the production of IL-10 by human monocytes. Monocytes from healthy donors were purified from theuffy coat and cultured for 24 h in the presence of Tat at 1, 10, 100, or 500 nM Tat or LPS at 100 ng/ml. No IL-10 production was detected in the absence of Tat or LPS (Fig. 1A, 1B). Tat and monocytes cultured in the absence of Tat or LPS (Fig. 1C) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and separated proteins were transferred to nitrocellulose membranes. Immunoblotting was conducted using either rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr-202/Tyr-204) antibody (1:1,000) (New England Biolabs, Hertfordshire, England) or rabbit anti-p44/42 MAP kinase antibody (1:1,000) (New England Biolabs). Membranes were incubated with the primary antibody (2 h at room temperature). Immunoreactive bands were then detected by incubation for 2 h at room temperature with swine anti-rabbit immunoglobulins conjugated with horseradish peroxidase (1:1,000) (Dako A/S, Roskilde, Denmark). The membranes were then visualized using a chemiluminescent substrate (Pierce, Rockford, Ill.).

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**FIG. 1. Production of IL-10 by monocytes treated with Tat.** (A) Monocytes (10⁶) were incubated in the absence or presence of Tat (1, 10, 100, or 500 nM) or LPS (100 ng/ml) for 24 h. (B) Monocytes were identically treated with Tat (10 or 100 nM) but for 24, 48, or 72 h. (C) Specificity of Tat. Monocytes (10⁶) were incubated in the absence or presence of native Tat (10 or 100 nM), oxidized Tat (1 h at 25°C in PBS plus 3% H₂O₂ at 10 and 100 nM), or LPS (100 ng/ml) for 24 h. (D) PBMC were depleted of monocytes by three successive adherence steps (1°, 2°, and 3°) in 24-well plates. After each adherence step, cells remaining in suspension (10⁶) were incubated in the absence or presence of LPS at 100 ng/ml or Tat at 1, 10, or 100 nM. Culture supernatants were recovered, and the presence of IL-10 was determined by ELISA. For A, B, and D, the values are the means ± standard deviation (SD) of three experiments with cells from one donor. Similar results were obtained with cells from three different donors. For C, the values are from results obtained with three different donors. Ctr, control.
mutant was used as a control. When tested in the same conditions, oxidized Tat was unable to induce IL-10 production by human monocytes (Fig. 1C). This specificity was further characterized using a mixture of three monoclonal antibodies directed against Tat. Preincubation of Tat (10 nM) with these antibodies totally inhibits IL-10 production (Fig. 2B).

As expected, LPS used at 100 ng/ml also induced high IL-10 production (Fig. 1A). Thus, the recombinant protein Tat was tested to detect possible contamination by this component. The Tat preparation used in this work contained no LPS within the limit of sensitivity of the test. Furthermore, LPS at the limit of detection in this test, 50 pg/ml, does not cause the production of IL-10 by monocytes in our system (data not shown). The production of IL-10 by monocytes is thus due to HIV-1 Tat.

Since our experiments were run with monocytes obtained from the buffy coat, the induction of IL-10 production was also confirmed with monocytes isolated from the fresh whole blood of healthy donors (data not shown). In order to determine if cells other than monocytes from peripheral blood can produce IL-10 following stimulation by Tat, monocytes in the PBMC population were depleted by several adherence steps and treated with Tat in the same conditions. The monocyte-free nonadherent cells did not produce IL-10 after treatment with 1, 10, and 100 nM Tat (Fig. 1D), indicating the direct implication of monocytes in the production of IL-10.

To determine the Tat region implicated in IL-10 production, we used first exon-deleted mutants GST-Tat 1-72 (RGD domain deleted), GST-Tat 1-55 (RGD and glutamic domains deleted), GST-Tat 1-45 (RGD, glutamic, and basic domains deleted), GST-Tat 20-72 (N-terminal domain deleted), and GST-Tat 30-72 (cysteine-rich region deleted). Results show that the C-terminally deleted mutants GST-Tat 1-72, GST-Tat 1-55, and GST-Tat 1-45 induced the same amount of IL-10 as the wild-type GST-Tat 1-101. Weak stimulation was observed with GST-Tat 20-72 (10 nM), while no stimulation was observed with GST-Tat 30-72 or with GST alone (Fig. 2A). These results indicate that the critical region responsible for the stim-
ulation was located within residues 1 to 45. In a second approach, we used three anti-Tat MAbs recognizing epitopes located within regions 1 to 15, 46 to 60, and 74 to 86. Preincubation of Tat with MAb 1-15 greatly inhibited (80.7%) the capacity of Tat to induce IL-10 production (Fig. 2B). Only a weak inhibition was obtained with Tat MAb 46-60, and no inhibition was observed with MAb 74-86. Thus, in agreement with the results with Tat recombinant mutants, the N-terminal region of Tat, amino acids 1 to 45, seems to be crucial for IL-10 stimulation.

Tat contains a nuclear localization sequence, and so there are two possible levels of action, the membrane and the nucleus. Tat was immobilized in wells in order to test whether it must penetrate monocytes to induce IL-10 production. In these conditions, monocyte stimulation by increasing concentrations of immobilized Tat led to dose-dependent production of IL-10 (Fig. 3A). In order to rule out the possibility that under these conditions some Tat entered the monocyte and induced IL-10 by an intracellular mechanism, Tat transactivation activity was evaluated in a comparative assay, depending on the intracellular localization of Tat, using HeLa P4 cells cultured with immobilized or soluble Tat. Tat immobilized in these conditions was unable to transactivate the HIV LTR, contrary to soluble Tat added at the same concentrations (Fig. 3B). Thus, Tat coated on the wells did not enter the cells, at least at the limit of sensitivity of the test. This suggests that Tat probably mediates its effect by direct interaction with the cell membrane. On the other hand, using flow cytometry analysis, we have shown, in agreement with previous reports (2, 21, 27, 49), that fluorescein isothiocyanate (FITC)-labeled Tat was able to bind to the cell membrane in a dose-dependent manner (data not shown).

Calcium pathway seems not to be involved in the production of IL-10 induced by Tat. We then sought to determine the signal transduction pathways involved in this production of IL-10. Two predominant signaling pathways known to induce the expression of cytokine genes were studied, the calcium pathway and the PKC pathway. These pathways are activated after stimulation of a membrane receptor that activates PLC, an enzyme that hydrolyzes phosphatidylinositol biphosphate to inositol 1,4,5-triphosphate, which initiates the calcium pathway, and to diacylglycerol, which activates PKC.

We initially used U73122, an inhibitor of PLC, to determine if this enzyme, the starting point for these signaling pathways, was involved in the activation by Tat. Significant inhibition (36 and 61.2%) of Tat-induced IL-10 production was observed when U73122 was used at 2.5 and 7.5 μM, respectively (Fig. 4). This suggests that the PLC pathway is involved in Tat-induced IL-10 production.

The calcium pathway is initiated by the presence of IP3, responsible for mobilizing intracellular calcium stores and thus the increased concentration of intracellular calcium. This pathway leads to the activation of calcineurin, a phosphatase that dephosphorylates transcription factor NF-AT. This enables the factor to undergo translocation to the nucleus, where it binds to specific sites on gene promoters, especially those coding for cytokines (47).
Two complementary approaches were used to determine the involvement of the calcium pathway in IL-10 induction by Tat, the first of which involved inhibitors. The compounds used were cyclosporin A (0.1 and 1 μg/ml), which inhibits calcineurin and acts by sequestering phosphorylated NF-AT in the cytoplasm, and BAPTA/AM (1.5 or 15 μM), a chelator of intracellular calcium. These two compounds did not significantly modify the Tat-mediated production of IL-10 by monocytes (Fig. 5A). As controls, we verified that the concentrations of cyclosporin A and BAPTA/AM used are biologically active (Fig. 5B). To this end, monocytes were stimulated with the calcium ionophore ionomycin, and production of tumor necrosis factor-alpha (TNF-α) was measured in the presence of these inhibitors. The results show that TNF-α induced by ionomycin was totally inhibited by cyclosporin A and markedly reduced (81%) by BAPTA/AM (Fig. 5B).

In the second approach, the role of calcium in the production of IL-10 was investigated by following the variations in cytoplasmic free Ca^{2+} concentrations ([Ca^{2+}]_{i}) at the cellular level by microspectrofluorimetry with the fluorescent probe fluo3-AM. Monocyte stimulation by Tat (100 nM) led to a very slight increase in the intracellular calcium concentration (Fig. 5C) that was at the limit of significance. These results were the mean for 11 different cells from a single donor. Similar results were obtained with cells from two different donors.

**PKC is indispensable for Tat-induced production of IL-10.** Monocytes treated with RO31-8220 (2.5 or 5 μM), a specific inhibitor of PKC that competes with ATP, could no longer produce IL-10 after treatment with Tat. Inhibition by the PKC inhibitor was dose dependent, being partial (58%) at 2.5 μM and total at 5 μM (Fig. 6A). This result suggests that PKC plays an important role in the mechanism of induction of IL-10 by Tat. In order to rule out a possible interference with PKA sometimes observed with PKC inhibitors, a PKA inhibitor, H89, was used at 50 or 100 μM. In these conditions, H89 had no significant effect on Tat-induced production of IL-10 (Fig. 6A). The specific inhibitory effects of RO31-8220 and H89...
were tested in an alternative IL-10 production assay in which
the stimulation of IL-10 was mediated by the PKA pathway. To
this end, monocytes were treated with rolipram, a phosphodi-
esterase inhibitor known to induce IL-10 production via the
PKA pathway (20). H89 inhibits in a dose-dependent manner
the IL-10 production mediated by rolipram, and this inhibition
became total at 100 \( \mu M \). In contrast, no significant inhibition
was observed with the PKC inhibitor RO318220 (Fig. 6B).
Together these results argued for the specific effect of protein
kinase inhibitors used in this study and underlined the major
role of PKC in Tat-induced IL-10 production.

The involvement of the PKC pathway was further charac-
terized. Monocytes were depleted of PKC by a long treatment
(24 or 48 h) with PMA (a PKC activator) at 50 or 100 ng/ml.
Treated monocytes were then incubated with Tat (10 nM) for
24 h, and the concentration of IL-10 was measured. The results
showed a strong inhibition of IL-10 production, up to 70% (Fig. 6C),
with monocytes treated with PMA for 24 h. Inhibi-
tion became total after 48 h of treatment with PMA (Fig. 6D).
Residual production of IL-10 after 24 h of treatment would be
due to activation by PMA and not to activation by Tat, since it
was also detected in cells treated with PMA alone (Fig. 6C).
In this procedure, PKC depletion was checked in experiments
involving restimulation of monocytes treated with PMA for 24
or 48 h. In these conditions of PMA restimulation, the cells
became unable to produce TNF-\( \alpha \) (data not shown). We can
note that there was no cell toxicity in these experimental con-
ditions, as shown by the trypan blue dye exclusion test. These
results strongly suggest that PKC plays an essential role in
the production of IL-10 by human monocytes after stimulation by
Tat.

We then attempted to investigate pathways activated down-
stream from the PKC by studying the known PKC substrate
transcription factor NF-\( \kappa \)B and the PKC-activated MAP ki-
nase ERK1/2 pathway.

**Involvement of transcription factor NF-\( \kappa \)B.** Transcription
factor NF-\( \kappa \)B is a likely candidate for the transactivation of
the IL-10 gene, since the organization of the IL-10 promoter shows
the presence of nine potential NF-\( \kappa \)B sites (23).

We examined the involvement of NF-\( \kappa \)B by first testing the
capacity of Tat to activate the nuclear translocation of this
factor with the electrophoretic mobility shift technique. These
experiments were done with an NF-\( \kappa \)B site and showed the
formation of a complex with nuclear extracts of monocytes
stimulated with 10 and 100 nM Tat (Fig. 7A, lanes 3 and 5).
The observed interaction between factor NF-\( \kappa \)B and the probe
seems to be specific, since no complex was observed when the
protein extract was incubated in the same conditions with the
mutated NF-\( \kappa \)B site (Fig. 7A, lane 4). Similar results were
obtained with nuclear proteins obtained from whole PBMC
(Fig. 7A, lanes 6 to 11) using phytohemagglutinin (PHA, 3 μg/ml) plus IL-2 (10 U/ml) as a positive control. On the other hand, no complex was detected when monocyte-depleted PBMC were treated with PHA plus IL-2 or with different concentrations of Tat (1, 10, and 100 nM) (Fig. 7A, lanes 12 to 16). To verify that NF-κB activation was specifically mediated by Tat, monocytes were treated with a chemically mutated Tat (oxidized Tat, used at 10 and 100 nM), and nuclear extracts were analyzed by EMSA. In agreement with the inability of this mutant Tat to stimulate the production of IL-10, no complex...
was detected in these conditions (Fig. 7B). These results indicate that Tat induces NF-κB activation specifically in monocytes. We then investigated if the region involved in IL-10 production was able to induce NF-κB activation. EMSA results show that only the C-terminally deleted mutants tested (GST-Tat 1-72 and GST-Tat 1-45) activate NF-κB as the wild-type GST-Tat 1-101 does (Fig. 7C, lanes 3 to 5). In contrast, no activation was observed with GST-Tat 20-72, GST-Tat 30-72 (Fig. 7C, lanes 6 and 7), or GST (Fig. 7C, lane 2). This result shows that NF-κB activation is correlated with the ability of Tat and Tat mutants to mediate IL-10 production.

We next tested the role of NF-κB activation in the production of IL-10. Monocytes were treated with nontoxic doses (50 and 100 μM) of TLCK and stimulated by Tat at 10 nM. NF-κB activation and IL-10 production were analyzed by EMSA and ELISA, respectively. The results depicted in Fig. 8A and 6B clearly showed that TLCK prevents both NF-κB translocation (Fig. 8A, lanes 7 and 8) and IL-10 production (Fig. 6B). These results indicate that the Tat-induced IL-10 production is correlated with Tat-induced NF-κB activation in monocytes. Tat thus activated transcription factor NF-κB, one of the substrates of PKC, thereby causing induction of the IL-10 gene.

Involvement of MAP kinases ERK1/2. MAP kinases p42 and p44, also called ERK1 and ERK2, can be activated by PKC (11, 12, 14, 51). This activation occurs after their phosphorylation by a cascade of kinases (Raf, MEK, and ERK) initiated by PKC. Once ERK1/2 are phosphorylated, they can activate transcription factors that bind to the promoters of cytokine genes.

In order for MAP kinases p42 and p44 to be activated, they must be phosphorylated on their tyrosine and threonine residues. The extent of activation of p42 and p44 was determined by treating monocytes with Tat (10 or 100 nM) for 15 or 30 min. Immunoblotting of the cytoplasmic extracts was then carried out, initially with an antibody against total p42 and p44 and then with an antibody against phosphorylated tyrosine and threonine residues. Western blots immunolabeled by specific antibodies against the phosphorylated residues showed a nonsignificant activation of ERK1/2 in cells treated with Tat (10 and 100 nM) after 15 min of stimulation (Fig. 9A). In contrast, treatment of monocytes with Tat for 30 min (10 and 100 nM) allowed a dose-dependent activation of MAP kinases ERK1/2.

The amounts of total MAP kinases analyzed were equivalent in all the lanes (Fig. 9A). Tat thus activates MAP kinases ERK1/2 in human monocytes.

The involvement of MAP kinases ERK1/2 in the Tat-induced production of IL-10 was tested by using a specific inhibitor of these MAP kinases PD98 059. When monocytes were treated with PD98 059 (10 and 100 μM, IL-10 production was partially inhibited. Inhibition was significant and was 52% with 100 μM inhibitor (Fig. 9B). These results suggest that Tat also activates MAP kinases ERK1/2 and thereby contributes to the production of IL-10.

**DISCUSSION**

There are now a large number of arguments in favor of a role of Tat protein in immune disorders occurring during HIV infection. Tat has a direct involvement in the stimulation of viral replication (34), activation of the provirus, and overexpression of virus coreceptors CXCR4 and CCR5 (30). It also disturbs the equilibrium of the immune system, e.g., by inducing the apoptosis of T cells (26), by inhibiting the activity of superoxide dismutase (55), and by acting on the expression of
genes of numerous cytokines, both those that it activates, such as IL-2 (44), IL-4, IL-8, IL-6, IL-1β, transforming growth factor beta, TNF-α, and TNF-β (26, 48) and those that it inhibits, such as IL-12 (45). IL-12 is a key cytokine that causes the differentiation of precursor T cells to TH1 cells (43).

In the present work, we have shown that HIV-1 Tat induces the production of IL-10 by human peripheral blood monocytes. The analysis of the signal transduction pathways showed that the calcium pathway is not or is only slightly involved; the PKC pathway apparently plays an essential role in the production of IL-10; transcription factor NF-κB, one of the main targets of PKC, is activated and is involved in this Tat-induced production of IL-10; and in parallel to the activation of NF-κB, MAP kinases ERK1/2 are also partially involved in the production of IL-10.

Tat also induces the production of TNF-α by human monocytes (15 and data not shown). It has been reported that TNF-α can potentiate the production of IL-10 (24). It is important to note, however, that TNF-α alone cannot induce the production of IL-10 by human monocytes (24). In agreement with these results, the addition of a neutralizing anti-TNF-α antibody in our work did not block the production of IL-10 (not shown).

Prior immobilization of Tat in the culture wells to prevent its intracellular penetration also allowed the stimulation of IL-10 production. This indicates that the interaction of Tat with a membrane receptor suffices to induce the production of IL-10 by human monocytes. Different regions of Tat have been implicated in interactions with membrane proteins: the N-terminal region with receptor CD26 (27), the tripeptide RGD (arginine-glycine-aspartate) with integrins αvβ3 and αvβ1 of dendritic cells (58), and the basic region with membrane lipids (49) or with the vegetative epidermal growth factor receptor of endothelial cells (2). Among this panoply of potential Tat receptors, it would be interesting to determine the receptor(s) that participates in the transduction of the signal leading to the production of IL-10 after stimulation by Tat. However, the observation that oxidized Tat is unable to mediate the production of IL-10 by monocytes suggests the importance of the cysteine-rich domain in the activity of Tat to stimulate IL-10 induction. To map the Tat domain implicated in this activity, the use of both recombinant Tat mutants and MAbs directed
against the N-terminal, central, or C-terminal region of Tat showed the involvement of N-terminal residues 1 to 45 of Tat in this activity. It is interesting to note that the implication of the N-terminal region, which contains the cysteine-rich domain but not the basic domain (responsible for penetration and nuclear localization of Tat), is in agreement with (i) the absence of activity of the cysteine-oxidized Tat and (ii) the fact that the effect of Tat is exerted at the membrane level.

It has recently been reported that Nef protein (9) and the transmembrane glycoprotein gp41 (5) also induce the production of IL-10. Comparison of these results with ours suggests that Tat operates via a signaling pathway different from those used by Nef and gp41. Nef apparently uses the calcium/calmodulin phosphodiesterase pathway. In the presence of W7, an inhibitor of this phosphodiesterase, or in the presence of EGTA, a calcium chelator, the induction of IL-10 is inhibited (9). In the case of Tat, on the contrary, the calcium pathway is not or is only very slightly involved, as shown by the absence of inhibition by cyclosporin A, an inhibitor of calcineurin, and BAPTA/AM, a chelator of intracellular calcium. These results obtained with calcium pathway inhibitors are consistent with microspectrofluorimetry determinations, at the cellular level, of variations in intracellular calcium concentrations. In addition, it has been reported that Tat blocks L-type calcium channels in dendritic cells (45) and NK cells (59). In contrast to these studies, it has been reported that synthetic Tat can induce a calcium signal in monocytes at concentrations from 6.6 to 33 nM (1). This effect was also observed with a peptide (CysL24,S31) containing the cysteine-rich region and the core region (1). Confocal microscopy will lead to a finer determination of the existence of possible variations in calcium concentrations in different cell compartments.

The induction of IL-10 by gp41 of HIV-1 rather seems to involve adenylate cyclase and cAMP (5). Sequences of the promoter of the IL-10 gene contain a cAMP-responsive element for transcription factors activated by cAMP. This pathway involves PKA and apparently is not involved in the mechanism of induction of IL-10 by Tat, since H89, a PKA inhibitor, has no effect on this production. In contrast, PKC activation is essential because the PKC inhibitor RO318220 inhibits the Tat-induced production of IL-10 in a dose-dependent manner; PKC depletion of monocytes by treatment with PMA abolishes the Tat-induced production of IL-10; and PMA, a direct activator of PKC, induces the production of IL-10. Thus, in contrast to the mechanisms used by Nef and gp41, the induction of IL-10 by Tat does involve the PKC pathway. This difference in signal transduction between Nef, gp41, and Tat can be partially explained by the nature of the membrane receptors involved, upstream, in the ligand-receptor interaction.

In the activation cascade involving PKC, we have thus demonstrated the activation of NF-kB, which is translocated into the nucleus as shown by gel mobility shift. We have also demonstrated the activation of the MAP kinases ERK1/2 pathway, leading to the activation of transcription factors, including AP-1, known for its involvement in the induction of several genes, including those of cytokines (25). It has also been reported that Tat produced endogenously in the U937 monocyte cell line can induce c-Jun N-terminal kinase (33), another member of the family of MAP kinases. It nevertheless remains to be determined if activation is accompanied by the modulation of expression of certain cellular genes.

The induction of inflammatory and immunomodulating cytokine genes (26, 44, 48), as well as the chemotaxis that can be exerted by Tat (1), suggests an activating role in the immune response. In spite of this, the overall effect of Tat in patients infected by HIV-1 appears rather suppressive. This can be explained by the inhibition of the proliferative T response (52, 56), the induction of T-lymphocyte apoptosis (26), the inhibition of phagocytosis of apoptotic bodies by dendritic cells (58), the cytotoxic activity of NK cells (59), and finally the suppression of IL-12 production by dendritic cells (45) and monocytes (31). The effect of Tat on IL-10 production reported here agrees with all of these reports concerning the potential immunosuppressive role of Tat during infection by HIV.

IL-10 is a cytokine produced by monocytes/macrophages, B cells, and T lymphocytes that suppresses cell-mediated immunity (41). IL-10 acts at different levels and is able to inhibit macrophage activity and to suppress the production of cytokines such as IL-1, TNF-α, and granulocyte-macrophage colony-stimulating factor (41). IL-10 also inhibits the proliferation of T lymphocytes by reducing major histocompatibility complex class II molecule expression on the surface of monocytes (41).

In summary, we have identified a signal transduction pathway used by Tat that leads to the production of IL-10 by human monocytes. Tat acts on membranes to initiate the activation of PKC, a key protein that can mobilize and activate NF-kB and also the members ERK1/2 of the MAP kinase family. NF-kB and other transcription factors activated by ERK1/2 are likely implicated in IL-10 gene induction. The multiple effects of IL-10 could contribute to the course of HIV infection to AIDS. The understanding of the molecular and cellular mechanisms involved in the production of this cytokine, by the identification of new specific targets, may suggest possible targeted therapeutic approaches to neutralize these effects.

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