Effects of HIV-1 Tat Protein on Ion Secretion and on Cell Proliferation in Human Intestinal Epithelial Cells

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Background & Aims: **Severe diarrhea and enteropathy of unknown origin are frequent in patients infected with human immunodeficiency type 1 virus (HIV-1). The HIV-1 transactivating factor protein (Tat) is a key factor in the pathogenesis of acquired immunodeficiency syndrome. We investigated whether Tat could directly induce ion secretion and cell damage in enterocytes.** ***Methods:*** **Electrical parameters (ion transport studies) were measured in Caco-2 cell monolayers and in human colonic mucosa specimens mounted in Ussing chambers. The effect of Tat on intestinal mucosa integrity was determined by monitoring the transepithelial electrical resistance of Caco-2 cell monolayers. ^{3}H-thymidine incorporation and cell count were used to evaluate the effect of Tat on cell growth. Intracellular calcium concentrations were measured at the single-cell level using microfluorometry technique.** ***Results:*** **Tat protein induced ion secretion in Caco-2 cells and in human colonic mucosa similar to that induced by bacterial enterotoxins. It also significantly prevented enterocyte proliferation. In both instances, the effect of Tat was maximum at concentrations within the range detected in the sera of HIV-1–infected patients. Anti-Tat antibodies inhibited both effects. Ion secretion and the antiproliferative effects were mediated by L-type Ca^{2+} channels. An increase in intracellular calcium concentration in Caco-2 cells was found after addition of Tat.** ***Conclusions:*** **These results indicate that Tat may be involved in HIV-1–related intestinal disease through direct interaction with enterocytes.**

Intestinal diseases are a hallmark of human immunodeficiency virus type 1 (HIV-1) infection. During disease progression, chronic diarrhea, dehydration, and malabsorption lead to progressive weight loss, which contributes to morbidity and mortality in HIV-1–positive individuals.[1] The cause of diarrhea is unknown in at least one third of cases, and it has been suggested that HIV-1 itself could cause diarrhea and intestinal damage.[2] Partial villus atrophy associated with a maturational defect of enterocytes (HIV enteropathy) has been reported in HIV-1–infected patients.[2,3] HIV-1–induced enteropathy is supported by the detection of viral proteins and/or nucleic acids in enterochromaffin as well as epithelial and goblet cells in the intestinal mucosa.[3] Infection of enterocytes by HIV-1, shown in vitro, could be a direct cause of enteropathy.[4] However, HIV-1 is not invariably detected in the intestinal epithelium of patients with acquired immunodeficiency syndrome, and diarrhea and malabsorption are not always correlated with the presence of HIV-1 in gut mucosa.[2] Consequently, whether or not HIV-1 plays a direct role in enteropathy remains controversial.

Several effects of HIV-1 are not mediated by the lytic propagation of viral particles but rather by secreted viral factors. For example, the bulk of T cells do not respond properly to antigenic or mitogenic stimuli even when only a small number of T cells are infected.[5] Abnormalities of proliferation and sometimes "bystander" apoptosis of uninfected cells have been associated with HIV-1 infection.[6] Thus, HIV-1 may alter the morphologic and functional maturation of intestinal epithelial cells without necessarily infecting enterocytes.[7] A viral toxin–like effect has been postulated for rotavirus infection, in which diarrhea is caused at least in part by the nonreplicating viral particle NSP4.[8,9]

In addition to structural and enzymatic proteins, HIV-1 encodes a group of at least 6 auxiliary regulatory proteins, including Tat, a transactivating peptide essential for HIV-1 replication, which exerts its effect by activating L-type Ca^{2+} channels[5,10,11] and/or by mobilizing intracellular calcium stores.[12] Despite its nuclear

Abbreviations used in this paper: BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid/acetoxymethyl ester; FCS, fetal calf serum; HIV, human immunodeficiency virus type 1; Isc, short-circuit current; NPPB, 5-nitro-2-3-(3-phenylpropylamino)benzoic acid; TEER, transepithelial electrical resistance.

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localization, Tat is secreted from HIV-1–infected cells and is taken up by neighboring uninfected cells. Tat is found in the sera of patients with acquired immunodeficiency syndrome in the absence of a massive lysis of infected cells[5] and is involved in many pathologic processes that may contribute to immune and nonimmune dysfunctions associated with HIV-1 infection.[5]

The aim of this study was to evaluate whether Tat alters transepithelial ion transport and/or induces cell damage in enterocytes. We conducted experiments with the Caco-2 cell line, which has the characteristics of mature human enterocytes, and with human colonic specimens obtained from patients undergoing surgery. Caco-2 cells have been used to investigate the enterotoxic and cytotoxic effects of toxins produced by enteric agents.[13,14] We also examined the effect of Tat on epithelial integrity and cell growth because villus atrophy in HIV enteropathy is frequently unassociated with compensatory crypt cell proliferation.[2]

Materials and Methods

Reagents and Cell Culture

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Chemically synthesized, high-performance liquid chromatography 96% pure HIV-1 Tat, as well as rabbit polyclonal antibody anti-Tat were purchased from Tecnogen (Piana di Monteverna, Italy). Bay K8644 was purchased from Calbiochem (La Jolla, CA). ^{3}H-thymidine was obtained from Amersham Italia (Milan, Italy). Culture media were from Life Technologies/GIBCO BRL (Mascia e Brunelli, Milan, Italy). Transwell filters and supports were from Costar (Costar Italia, Milan, Italy). Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle medium with a high glucose concentration (4.5 g/L) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, penicillin (50 mU/mL), and streptomycin (50 mg/mL) and were incubated in 5% CO_2/95% air. The medium was changed daily.

Ion Transport Studies

Caco-2 cells were grown on uncoated polycarbonate Transwell filters and used for intestinal transport studies 15 days after confluence as previously described.[13] The filter area was 4.9 cm^2. Each filter was mounted in an Ussing chamber (WPI, Sarasota, FL) as a flat sheet between the mucosal and serosal compartments. Each compartment contained 5 mL of Ringer's solution with the following composition (in mmol/L): 114 NaCl, 5 KCl, 1.65 Na_2HPO_4, 0.3 NaH_2PO_4, 1.25 $CaCl_2$, 1.1 $MgCl_2$, 25 $NaHCO_3$, and 10 glucose; the buffer was constantly gassed with 95% O_2/5% CO_2 and was connected to a thermostat-regulated circulating pump to maintain a temperature of 37°C.

The following electrical parameters were measured as described elsewhere[15] before and after the mucosal or serosal addition of Tat: transepithelial potential difference, short-circuit current (Isc), and tissue conductance. Isc is expressed as microamperes per square centimeter, tissue conductance as milliseconds per square centimeter, and potential difference as millivolts. Cell viability was evaluated by measuring the electrical response to the serosal addition of theophylline (5 mmol/L) at the end of each experiment.

In experiments performed to investigate the role of Cl^- in the electrical response, SO_4^- substituted Cl^- at an equimolar concentration. To investigate in greater detail the role of Cl^- in the electrical effect of Tat, we used the Cl^- channel inhibitor 5-nitro-2-3-(3-phenylpropylamino)benzoic acid (NPPB) as previously described.[14] Cells were incubated with NPPB (100 nmol/L), Tat was added later, and electrical parameters were monitored. Experiments were performed to investigate the role of Ca^{2+} in the effects of Tat by use of membrane-permeant 1,2-bis(2-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) as previously reported.[14] Caco-2 cells were pretreated with 20 μmol/L BAPTA/AM on both the mucosal and serosal sides; 30 minutes later, Tat (0.1 nmol/L) was added and electrical parameters were measured. Parallel monolayers treated with BAPTA/AM alone or Tat alone served as controls.

Bay K8644, a specific agonist of L-type Ca^{2+} channels, was used to investigate the role of L-type Ca^{2+} channels in the electrical effects exerted by Tat. Caco-2 cell monolayers were incubated for 20 minutes with Bay K8644 (1 μmol/L, on the serosal side), after which Tat (0.1 nmol/L) was added on the serosal side.

Neutralization experiments were performed using specific anti-Tat polyclonal antibodies developed in rabbit using purified synthetic Tat protein as immunogen. Tat (0.1 nmol/L) was incubated at 37°C for 1 hour with the antibodies (6 ng/mL) and then added to Caco-2 cells in Ussing chambers. The same concentration of preimmune antibodies was incubated with Tat and used as controls.

Experiments on human intestine were performed using colonic specimens obtained from 4 patients (all men; mean age, 38 ± 11 years) undergoing surgery because of colon cancer. The specimens were obtained from the edges of resected margins and appeared histologically normal.

All specimens came from a segment located within 15 cm of the ileocecal valve. Intestinal tissue was kept in ice-cold saline solution, stripped of the serosal and muscular layers, and mounted in Ussing chambers within 15 minutes of surgical excision.[16] Four paired fragments of stripped proximal colonic mucosa were mounted in Ussing chambers. In each experiment, one paired fragment served as a control of baseline electrical parameters. The bathing Ringer's solution contained the following (in mmol/L): 53 NaCl, 5 KCl, 30.5 Na_2SO_4, 30.5 mannitol, 1.69 Na_2HPO_4, 0.3 NaH_2PO_4, 1.25 $CaCl_2$, 1.1 $MgCl_2$, and 26 $NaHCO_3$. The viability of intestinal specimens mounted in each Ussing chamber was checked at the end of each experiment by evaluating the electrical response to the addition of theophylline (5 mmol/L) to the serosal side.

The study protocol was approved by the ethics committee of the School of Medicine at the University Federico II of Naples.

Determination of Intracellular Cyclic Nucleotide Concentrations

Adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) concentrations in Caco-2 cells were determined with commercial kits (Biotrak cyclic AMP and Biotrak cyclic GMP assay system; Amersham International, Amersham, United Kingdom) as previously reported.[14,17]

Determination of Intracellular Calcium Concentrations

$[Ca^{2+}]_i$ was measured using a microfluorometric technique as previously reported.[18] Briefly, cells grown on glass coverslips were loaded with 5 μmol/L fura-2 AM in Krebs–Ringer saline solution for 1 hour at 22°C. After loading, the coverslip was introduced into a microscope chamber (Medical System Co., Greenvale, NY) on an inverted Nikon Diaphot fluorescence microscope (Nikon Corp., Tokyo, Japan). Cells were kept in Krebs–Ringer saline solution throughout the experiment. All substances tested were introduced into the microscope chamber by fast injection. A 100-W xenon lamp (Osram, Frankfurt, Germany) with a computer-operated filter wheel bearing 2 different interference filters (340 and 380 nm) illuminated the microscopic field with UV light, alternating the wavelength at an interval of 500 milliseconds. The interval between each pair of illuminations was 2 seconds, and the interval between filter movements was 1 second. Consequently, $[Ca^{2+}]_i$ was measured every 3 seconds. Emitted light was passed through a 400-nm dichroic mirror, filtered at 510 nm, and collected by a charge-coupled device camera (Photonic Science, Robertsbridge, United Kingdom) connected to a light amplifier (Applied Imaging Ltd., Dukesway Gateshead, United Kingdom). Images were digitized and analyzed with a Magiscan image processor (Applied Imaging Ltd.). Using a calibration curve, AUTOLAB software (RBR Altair, Florence, Italy) was used to calculate the $[Ca^{2+}]_i$ corresponding to each pair of images from the ratio between the intensity of the light emitted when cells were illuminated at both 340 and 380 nm. At the end of each experimental session, the calibration was performed according to the procedure described by Grynkiewicz et al.[19] In particular, cells were lysed with ionomycin (2–10 μmol/L) in the presence of 1.5 mmol/L extracellular Ca^{2+}. Addition of ionomycin produced a rapid increase in fluorescence intensity that allowed us to calculate the maximal ratio value. To determine the minimal ratio value, cells were subsequently exposed to a Ca^{2+}-free solution containing 1–20 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. Given that the dissociation constant for Ca^{2+} of fura-2 AM is 224 nmol/L at 37°C, the minimal ratio and maximal ratio values were introduced into the Grynkiewicz formula to convert the values of fluorescence ratio between 340 and 380 nm into $[Ca^{2+}]_i$. The values were subtracted for background fluorescence obtained from images taken from a region of the coverslip devoid of cells. No interference was detected between any of the compounds used in the present study and the excitation or the emission spectra of fura-2 AM.

Epithelial Integrity Studies

We measured the transepithelial electrical resistance (TEER) of cell monolayers grown on polycarbonate Transwell filters. Each filter was seeded with 2×10^6 cells, and experiments were performed 15 days after confluence as previously reported.[20] Tat was added to the mucosal or serosal side of the cell monolayer, and TEER was monitored using a resistance-monitoring apparatus (Millicel-ERS; Millipore, Milan, Italy) as previously described.[20] A positive control was provided by a rotavirus strain SA11–induced decrease in TEER as described elsewere.[20] Resistance was expressed in ohms per square centimeter.

Cell Growth Studies

To test the hypothesis that Tat inhibits intestinal cell growth, we used 2 experimental models: ^{3}H-thymidine incorporation and cell counting.

^{3}H-thymidine incorporation. Cells were seeded into 96-well microtiter plates (10^4 cells/well) in Dulbecco's modified Eagle medium with 10% FCS and incubated for 12 hours to allow entry into growth phase. The cells were incubated with FCS-free Dulbecco's modified Eagle medium and with the testing substances for a further 30 hours. ^{3}H-thymidine (0.5 μCi/well) was added and incubation was continued for 18 hours. Cells were harvested with a Titertek cell harvester (Flow Laboratories, Rickmansworth, United Kingdom); the resulting filters were dried and beta radioactivity was counted with a Packard scintillation spectrometer.

Cell counting. Cells were plated into 24-well tissue culture plates (10^4 cells/well) using 2 wells for each experimental condition and were grown in Dulbecco's modified Eagle medium supplemented with 10% FCS and antibiotics for 72 hours. Cells were then deprived of serum for 24 hours. Cells were harvested with 1% trypsin 48 hours after the addition of each testing substance. The resulting cell suspensions were randomized to another investigator, and cells were counted in a blinded fashion. Cell viability was determined by trypan blue exclusion. The difference in paired counts did not exceed 10%.

We investigated the role of L-type Ca^{2+} channels in the effect caused by Tat using both ^{3}H-thymidine incorporation and cell counting in the presence of the specific agonist Bay K8644 (1 μmol/L). Bay K8644 was added to the medium 1 hour before the addition of Tat and remained in the incubation medium throughout the experiments (i.e., for 48 hours).

Neutralization experiments were performed using specific anti-Tat polyclonal antibodies. Tat (0.1 nmol/L) was incubated in FCS-free medium containing 0.2% BSA at 37°C for 1 hour with the antibodies (10 ng/1 ng of Tat) before being added to Caco-2 cells. The same concentration of preimmune antibodies was used in paired controls.

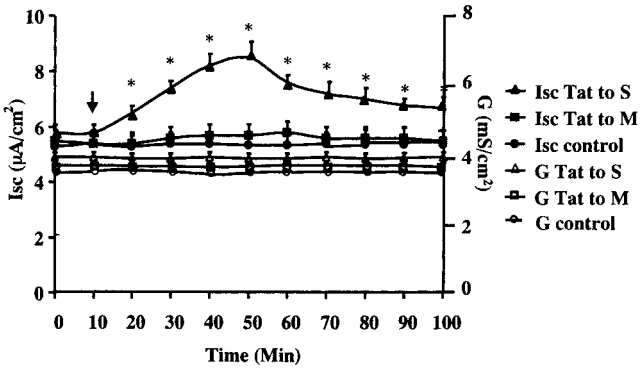

Figure 1. Time course of the effect on Isc and monolayer electrical conductance (G) of Tat (0.1 nmol/L) added to the serosal (S) or mucosal (M) side of Caco-2 cells mounted in Ussing chambers. The *arrow* indicates the time of Tat addition. The number of observations is 9 for each data point. Data are means ± SE. *$P < 0.01$ vs. control.

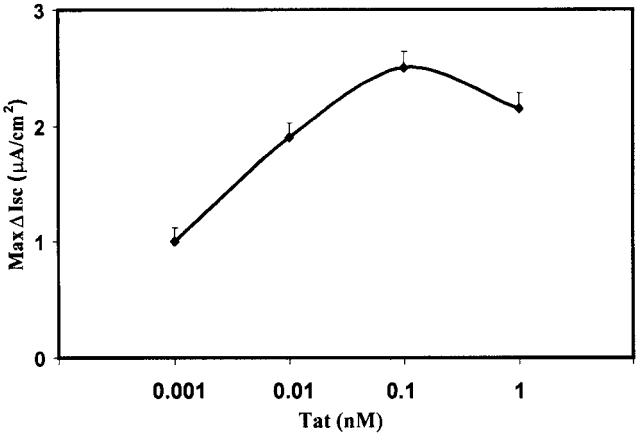

Figure 2. Isc modifications in response to increasing concentrations of Tat added to the serosal side of Caco-2 cells. ΔIsc values are expressed as the maximal difference between control cells and cells exposed for 60 minutes to Tat. The number of observations is 9 for each data point. Data are means ± SE.

Statistics

Data are expressed as means ± SE, and significance was evaluated by the nonparametric, 2-tailed Mann–Whitney *U* test. A *P* value <0.05 was considered significant. The SPSS software package for Windows (release 11.0.1; SPSS Inc., Chicago, IL) was used for statistical analysis.

Results

Effects of Tat on Transepithelial Ion Transport in Caco-2 Cells

The addition of Tat to the mucosal side of Caco-2 monolayers did not affect electrical parameters. In contrast, when added to the serosal side, Tat caused an enterotoxic-like secretory effect; it increased the Isc and potential difference but did not alter tissue conductance. The increase in Isc peaked approximately 40 minutes after the addition of Tat and was sustained. Isc did not return to baseline for 90 minutes (Figure 1).

The effect of Tat on Isc was dose dependent. It was detected at a concentration as low as 0.001 nmol/L and peaked at 0.1 nmol/L; higher doses did not increase Isc further, indicating that the enterotoxic effect was saturable (Figure 2).

To determine whether the enterotoxic effect was specific, we incubated Tat (0.1 nmol/L) in Ringer's solution at 37°C for 1 hour with increasing concentrations of specific anti-Tat polyclonal antibodies raised in rabbits and then added the solution to Caco-2 cells in Ussing chambers. Specific antibodies significantly inhibited the electrical effect of Tat (Table 1). Incubation with preimmune antibodies had no effect on Tat-induced increase in Isc.

To determine whether the electrical effect was caused by anion secretion rather than cation absorption, we performed the same experiments using Cl^--free Ringer's solution. Without Cl^-, the electrical effect was virtually abolished. Thus, the effect of Tat on Isc was entirely due to transepithelial Cl^- secretion (Table 1). To investigate in greater detail the role of Cl^- in the electrical effect of Tat, we added the protein to Caco-2 cells at concentrations capable of eliciting the maximal secretory response (0.1 nmol/L) in the presence of the Cl^- channel inhibitor NPPB as previously described.[14] NPPB completely inhibited the secretory effect of Tat (Table 1). Thus, the Tat-induced enterotoxic effect is related to the Cl^- channels.

Most enterotoxins that induce intestinal Cl^- secretion act by increasing cAMP and cGMP intracellular concentrations[21] or by modifying Ca^{2+} concentrations. To determine whether either cyclic nucleotide was involved in the Tat-induced enterotoxic effect, we measured cAMP and cGMP concentrations before and after exposure of

Table 1. Modifications of Isc by Tat in Various Experimental Conditions

Experimental conditions	Maximum Δ Isc
Tat alone (control)	2.50 ± 0.11
Tat in Cl^--free medium	0.09 ± 0.13[a]
Tat + NPPB	0.05 ± 0.12[a]
Tat + BAPTA/AM	0.20 ± 0.20[a]
Tat + Bay K8644	0.72 ± 0.15[a]
Tat + anti-Tat antibody	0.70 ± 0.06[a]

NOTE. Experiments were performed to investigate the mechanisms of the effects of Tat on ion transport, namely the involvement of Cl^- (either in Cl^--free medium or in the presence of NPPB), the involvement of Ca^{2+} (either in the presence of BAPTA/AM or Bay K8644), and the specificity of the effect (in the presence of anti-Tat antibody). The results are means ± SE.

[a]$P < 0.01$ vs. Tat alone.

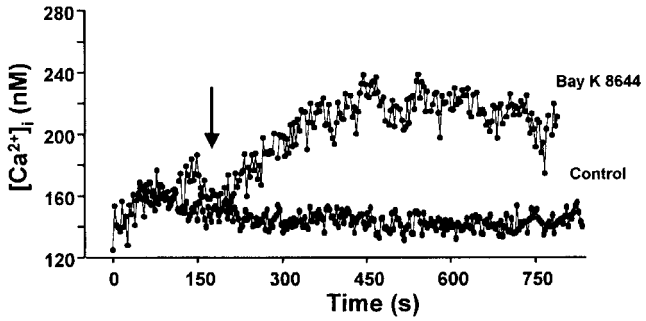

Figure 3. Functional demonstration of L-type Ca^{2+} channel expression in Caco-2 cells obtained with a fura-2 AM microfluorometric technique. A representative single-cell trace for the effect of Bay K8644 (1 μmol/L) in a Caco-2 cell is shown. The trace is representative of 20 cells for each experimental group studied in at least 3 different experimental sessions. The *arrow* indicates application of Bay K8644. The presence of the L-type Ca^{2+} channels in Caco-2 cells was shown by a sustained increase in $[Ca^{2+}]_i$ elicited by the addition of Bay K8644, the specific agonist of the L-type Ca^{2+} channels.

cell monolayers to the viral protein. Basal cAMP and cGMP concentrations were 2.1 ± 0.3 and 2.5 ± 0.2 pmol/mg cell protein, respectively, and were not modified by the addition of 0.1 nmol/L Tat (2.2 ± 0.1 and 2.5 ± 0.3 pmol/mg cell protein, respectively).

To investigate the involvement of intracellular Ca^{2+} in the Tat effect, cell monolayers mounted in Ussing chambers were preloaded with BAPTA/AM as described in Materials and Methods. The subsequent addition of Tat did not result in any increase in Isc, suggesting Ca^{2+} dependence of the secretory effect. No effect was observed with BAPTA/AM alone in control cells (Table 1).

To investigate the role of L-type Ca^{2+} channels in the secretory effects exerted by Tat, we incubated Caco-2 cell monolayers for 20 minutes with the specific agonist of L-type Ca^{2+} channels Bay K8644[22] (1 μmol/L, on the serosal side) and then added Tat (0.1 nmol/L). Bay K8644 induced a significant reduction of the effect, which indicates that the L-type Ca^{2+} channels are involved in the intestinal ion secretion induced by Tat (Table 1).

Modifications of Caco-2 $[Ca^{2+}]_i$ in Response to Tat

As shown in Figure 3, the addition of the specific L-type Ca^{2+} channel agonist Bay K8644 (1 μmol/L) to Caco-2 cells previously loaded with fura-2 AM indicator elicited a sustained increase in $[Ca^{2+}]_i$, suggesting an activation of specific L-type Ca^{2+} channels.

When Caco-2 cells were superfused with Tat protein (0.1 nmol/L), an increase in $[Ca^{2+}]_i$ was observed. This showed a bimodal pattern; 82% of responding cells displayed a progressive and sustained increase, whereas 18% of the cell population showed a single-spike transient increase (Figure 4*A*).

The Tat-induced increase in $[Ca^{2+}]_i$ was partially inhibited either in the absence of extracellular Ca^{2+} or in the presence of 10 μmol/L nimodipine, the specific inhibitor of L-type voltage-gated calcium channels (Figure 4*B*). This suggests that the Tat-induced increase in $[Ca^{2+}]_i$ involves either extracellular Ca^{2+} entrance or mobilization from intracellular stores.

Effects of Tat on Human Intestinal Ion Transport

To assess whether the secretory effect induced by Tat in Caco-2 cells was reproducible in human intestine,

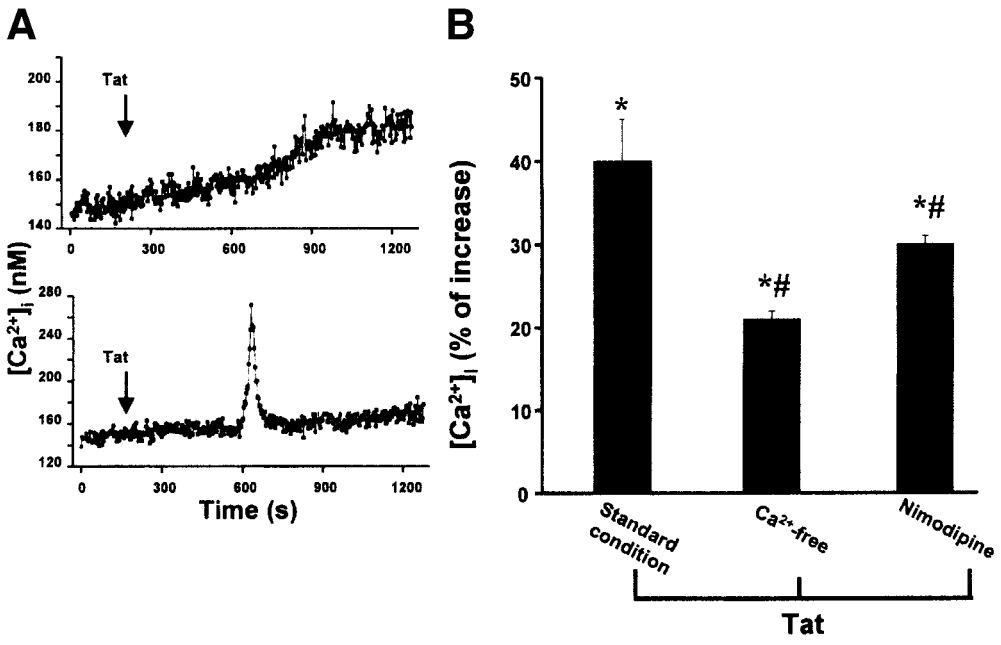

Figure 4. Effect of Tat (0.1 nmol/L) on $[Ca^{2+}]_i$ in Caco-2 cells obtained with a fura-2 AM microfluorometric technique. (*A*) Two profiles of a Tat-induced increase in $[Ca^{2+}]_i$ are observed in Caco-2 cells: one sustained (*upper panel*) and a distinct single-spike increase (*lower panel*). The traces are representative of 20 cells for each experimental group studied in at least 3 different experimental sessions. (*B*) The quantification of a Tat-induced increase in $[Ca^{2+}]_i$ in Caco-2 cells expressed as percentage of increase above baseline values in the standard condition or in Ca^{2+}-free solution or in the presence of nimodipine (1 μmol/L). $^*P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. Tat in standard condition.

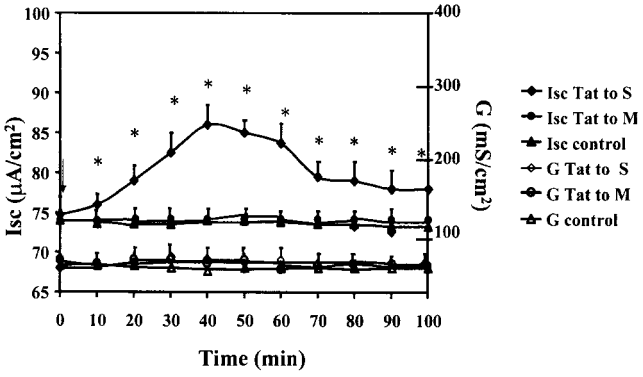

Figure 5. Effects of Tat on transepithelial ion transport in human colon. Time course of the effect on Isc and on electrical conductance (G) elicited by the addition of Tat (0.1 nmol/L) (*arrow*) to the mucosal or serosal side of human colonic mucosa mounted in Ussing chambers. Data are means ± SE. $*P < 0.05$ vs. control.

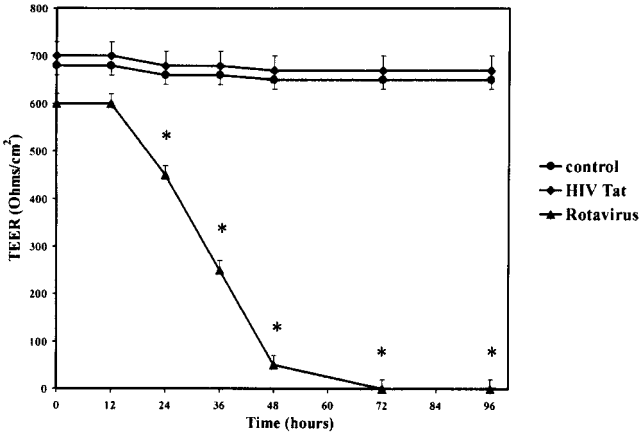

Figure 6. Effect of Tat on epithelial integrity. Changes in TEER of the Caco-2 cell monolayer in the presence of Tat (0.1 nmol/L) or rotavirus SA11 (5 PFU/cell). Data are means ± SE. $*P < 0.01$ vs. control.

we performed experiments using fragments of stripped proximal colonic mucosa mounted in Ussing chambers obtained from 4 different patients undergoing surgery. The serosal addition of Tat (0.1 nmol/L) to human colonic mucosa induced the same secretory effect obtained in Caco-2 cells: an increase in Isc entirely related to changes in potential difference without variations of tissue ionic conductance. Electrical parameters remained unchanged for as long as 100 minutes after the addition of Tat to the mucosal side of human colonic specimens (Figure 5).

Effects of Tat on Intestinal Epithelial Integrity

The Ussing chamber experiments showed that epithelial integrity was conserved in the short-term. To determine whether long-term incubation with Tat damaged the Caco-2 cell monolayer, we measured the TEER of cells grown on polycarbonate Transwell filters. With this experimental model, cytopathic effects may be monitored for several days. Cell damage is reflected by a decrease in TEER. A positive control was provided by the rotavirus strain SA11–induced decrease in TEER as previously reported.[20] The addition of rotavirus (5 plaque-forming units/cell) to the apical side of Caco-2 cells induced a progressive decrease in TEER (Figure 6). On the contrary, incubation for up to 96 hours (4 days) in the presence of a mucosal or serosal Tat concentration as high as 100 nmol/L did not cause any change in TEER (Figure 6).

Effects of Tat on Intestinal Epithelial Cell Growth

Continuous exposure of near-confluent Caco-2 cells to Tat for 48 hours dose-dependently inhibited incorporation of ^{3}H-thymidine (Figure 7). The maximal inhibition (>50%) was obtained with 0.1 nmol/L (i.e., the same concentration that induced the maximal effect on ion transport). Cell-counting experiments confirmed the results of ^{3}H-thymidine incorporation (Figure 7). The effect on cell growth was dose dependent and saturable. The minimal effect was observed with 0.001 nmol/L Tat.

We next investigated the role of L-type Ca^{2+} channels using ^{3}H-thymidine incorporation and cell counting in the presence of the specific inhibitor Bay K8644 (1 μmol/L). Bay K8644 was added to the medium 1 hour before the addition of Tat, where it remained throughout the experiment (48 hours). Bay K8644 alone did not significantly affect Caco-2 cell growth. On the contrary, it significantly inhibited the effect induced by Tat on cell growth. Neutralization experiments were performed in the presence of specific anti-Tat antibodies. In these conditions, the effects of Tat on cell growth were virtu-

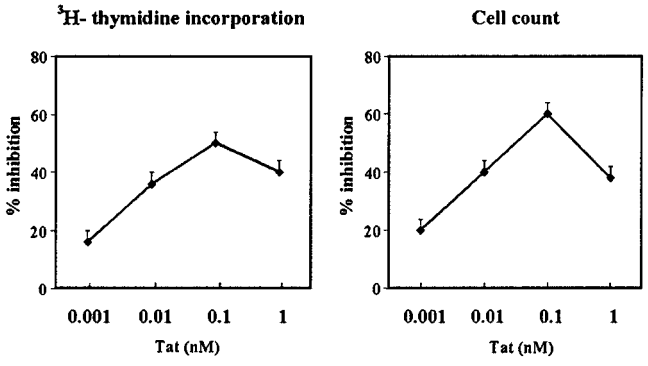

Figure 7. Effect of increasing concentrations of Tat on ^{3}H-thymidine incorporation and cell proliferation in Caco-2 cells. Data are means ± SE of 3 independent experiments.

ally abolished. Incubation with preimmune antibodies had no effect on the effect of Tat on cell growth.

Discussion

The results of our study show that Tat directly exerts pathogenic effects on enterocytes. Like a bacterial enterotoxin, Tat interacted with Caco-2 cells and induced ion secretion similar to that previously obtained in the same experimental model with other established secretagogues, including *Vibrio cholerae* toxin, *Escherichia coli* heat-stable enterotoxin, and *Cryptosporidium parvum*–associated enterotoxic activity.[16,17] The addition of Tat to human colonic mucosa resulted in the same electrical effect observed in Caco-2 cells (i.e., an increase in Isc indicating Cl^- secretion). The magnitude of the electrical response was similar to that observed with other Ca^{2+}-mediated secretagogues.[14] Tat exerted a polar-type effect (i.e., it resulted from interaction with the basolateral but not the apical cell membrane). The polarity of the effect has clinical relevance because Tat is released from HIV-1–infected cells in the sera of patients with acquired immunodeficiency syndrome.[5,23] Interestingly, the Tat concentration that induced the maximal secretory response in vitro was within the range of that generally measured in the sera of patients with HIV, suggesting that Tat concentrations effective in inducing intestinal ion secretion may well be reached in vivo.[23]

There was no evidence of cell damage in our experimental conditions. Tissue conductance and its reciprocal resistance are sensitive parameters of cell damage and remained unchanged in both short-term experiments in Ussing chambers and in long-term experiments in which TEER was measured. This suggests that enterocytes, in contrast to other cells such as neurons, are insensitive to the cytotoxic effect of Tat.[5] The lack of cytopathic effect could be due to a cell type–specific effect or the action of protective substances produced by intestinal cells in response to other intestinal pathogens.[24,25] However, Tat indirectly induced epithelial damage. When we incubated actively replicating cells with increasing Tat concentrations, both cell counts and ^{3}H-thymidine incorporation decreased, which reflects an antiproliferative effect. Similar to data from the ion transport studies, cell growth experiments showed that the effects of Tat resulted from direct interaction with enterocytes and suggest that Tat is involved in the pathogenesis of intestinal mucosal atrophy typical of HIV-1–infected patients.[2,26–28] Neutralization experiments with anti-Tat antibodies supported the specificity of these effects. Thus, Tat protein exerts a short-term effect on ion transport and a long-term effect on cell growth. Whether these apparently distinct effects are functionally related remains to be elucidated.

Interestingly, Tat and vascular endothelial growth factor show amino acid sequence homologies and may exert similar effects in human endothelial cells.[29] Recently, intestinal-specific receptors have been described[30] and an antiproliferative effect elicited by vascular endothelial growth factor in Caco-2 cells has been reported.[31] Thus, it is possible that some intestinal actions of Tat can be mimicked by peptides containing a similar amino acid sequence.

Several lines of evidence in this study indicated that the mechanisms of Tat enteropathogenic effect were Ca^{2+} dependent. Tat was able to induce an increase in $[Ca^{2+}]_i$ in Caco-2 cells, which are responsive to L-type Ca^{2+} channel agonists. Interestingly, Tat and Bay K8644, a specific L-type agonist that inhibited the effects of Tat in Caco-2 cells, compete for binding to dendritic cells,[32] which further supports the hypothesis that the effects of Tat involve L-type Ca^{2+} channels. Finally, BAPTA/AM prevented Tat-induced ion secretion, suggesting an effect dependent on $[Ca^{2+}]_i$ modifications.

The bimodal pattern of $[Ca^{2+}]_i$ increase in cell response observed in Caco-2 cells resembles that observed in neurons[12] exposed to Tat and suggests that distinct transduction mechanisms may be involved. The experiments performed with nimodipine and in the absence of extracellular Ca^{2+} suggested that a Tat-induced increase in $[Ca^{2+}]_i$ involves either the extracellular Ca^{2+} entrance or Ca^{2+} mobilization from intracellular stores. Similar mechanisms have been suggested for the effects of rotavirus enterotoxin NSP4.[9] It is somehow a striking finding that similar to NSP4, another nonstructural viral protein, Tat protein promotes a Ca^{2+}-mediated enterotoxic effect that is potentially involved in the pathogenesis of diarrhea in HIV-1–infected patients.

Intestinal dysfunction is a common feature of HIV infection,[33] and highly active antiretroviral therapy results in a rapid and significant improvement in intestinal function.[34] Similarly, combined antiretroviral therapy improves chronic HIV-1–related pathogen-negative diarrhea.[35] These data, together with the inverse correlation between natural anti-Tat antibody titers and disease progression,[5] suggest that circulating Tat is important in the pathogenesis of acquired immunodeficiency syndrome–related intestinal disease.

Finally, our results may have therapeutic implications. The finding that specific antibodies significantly inhibit the effects exerted in vitro by Tat on both ion secretion and cell proliferation suggests that interdiction of extra-

cellular Tat by active or passive immunization would reduce its pathogenic effects in the intestine. As recently proposed, a functionally inactivated but immunogenic Tat preparation (Tat toxoid) that triggers long-lasting Tat antibodies could be a candidate for a prophylactic or therapeutic vaccine. Such a preparation would prevent Tat-induced intestinal HIV-1 enteropathy.[36,37]

In conclusion, the addition of Tat to human enterocytes or human colonic mucosa induces electrolyte secretion similar to that caused by classical bacterial enterotoxins, a finding that supports the direct role of Tat in diarrhea. In addition, Tat induces a potent antiproliferative effect on enterocytes, thus implicating the protein in the pathogenesis of the intestinal mucosal atrophy typical of HIV-1–infected patients.

Taken together, these findings indicate that Tat may play a key role in HIV-1–associated intestinal disorders.

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