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The Binding Subunit of Pertussis Toxin Inhibits HIV Replication in Human Macrophages and Virus Expression in Chronically Infected Promonocytic U1 Cells

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We have recently shown that the binding subunit of pertussis toxin (PTX-B) inhibits the entry and replication of macrophage-tropic (R5) HIV-1 strains in activated primary T lymphocytes. Furthermore, PTX-B suppressed the replication of T cell-tropic (X4) viruses at a postentry level in the same cells. In this study we demonstrate that PTX-B profoundly impairs entry and replication of the HIV-1ADA (R5), as well as of HIV pseudotyped with either murine leukemia virus or vesicular stomatitis virus envelopes, in primary monocyte-derived macrophages. In addition, PTX-B strongly inhibited X4 HIV-1 replication in U937 promonocytic cells and virus expression in the U937-derived chronically infected U1 cell line stimulated with cytokines such as TNF-α and IL-6. Of interest, TNF-α-mediated activation of the cellular transcription factor NF-kB was unaffected by PTX-B. Therefore, PTX-B may represent a novel and potent inhibitor of HIV-1 replication to be tested for efficacy in infected individuals.

In support of this proposition, a genetically modified mutant of PTX (PT-9K/129G), which is safely administered for prevention of Bordetella pertussis infection, showed an in vitro anti-HIV profile superimposable to that of PTX-B. The Journal of Immunology, 2001, 166: 1863–1870.

Abbreviations used in this paper: PTX, pertussis toxin; R5, macrophage-tropic; X4, T cell-tropic; MDM, monocyte-derived macrophages; MS, multiply spliced; RT, reverse transcriptase; MuLV, murine leukemia virus; VSV, vesicular stomatitis virus; VSV-G, vesicular stomatitis virus G protein; LTR, long terminal repeat; US, unspliced; WT, wild-type; PT-9K/129G, a genetically modified mutant of PTX.
eradicating HIV-1 infection due to their long lifespan and relative absence of cytopathicity consequent to retroviral infection (27, 28). Although expressing both CCR5 and CXC chemokine receptor 4, monocyte-derived macrophages (MDM) sustain efficient replication of R5, but usually not of X4 HIV-1 due to restrictions occurring at a postentry level (29). However, promyelocytic or promonocytic cell lines such as HL-60 or U937, respectively, are readily infectable by X4 viruses and have been broadly used as models of acute viral infection of mononuclear phagocytic cells in vitro. In this context, the U1 promonocytic cell line is one of the most thoroughly characterized models of postintegration latency. This cell line was obtained from a population of U937 cells surviving the cytopathic effect of acute HIV-1 (X4) infection, and contains two copies of integrated proviruses (30, 31). In the absence of stimulation, U1 cells exhibit a pattern of viral mRNA expression characterized by low levels of multiply spliced (MS) 2-kb transcripts encoding the regulatory proteins Tat, Rev, and Nef (32). This pattern was interpreted early as an indication of a Rev-dependent restriction of HIV expression (32). However, several studies have subsequently demonstrated that the relative state of latency in U1 cells is a consequence of defective Tat rather than Rev function (33, 34). High levels of virus production can be rapidly induced by stimulation of U1 cells with proinflammatory cytokines, such as TNF-α and IL-6, among others (reviewed in Ref. 35), and by PMA (31). Of interest, these agents have been shown to activate HIV-1 expression in U1 cells by affecting distinct steps of the virus life cycle, including NF-κB-dependent transcription in the case of TNF-α and PMA (36, 37) and posttranscriptional event(s) in cells stimulated with IL-6 (38).

This study was designed to investigate the effect of PTX-B on HIV-1 infection of mononuclear phagocytes, both acutely and chronically infected with HIV-1. Our results demonstrate that PTX-B as well as a genetically modified PTX (PTK9/129G) potently inhibited HIV replication in primary MDM and cytokine-induced virus expression in chronically infected U1 cells. The latter activity is of special importance as it suggests a potential value of PTX-B as an agent that can prevent reactivation of latent HIV provirus for a major determinant of the rapid rebound of viral replication observed upon withdrawal of antiretroviral therapy in AIDS patients.

Materials and Methods

Reagents

PTX (Sigma, St. Louis, MO) and PTX-B (Calbiochem, San Diego, CA) were dissolved in sterile PBS and stored at 4°C; PT-9K/129G (Chiron, Emeryville, CA) was dissolved in 50% glycerol/0.5 M NaCl. The purity of PTX-B was tested by SDS-PAGE analysis, which failed to detect any contaminating holotoxin in PTX-B preparations. Based on previous observations (2, 39), both PTX-B and PTX were used at the final concentration of 1 nM unless otherwise indicated. Recombinant IL-6 and TNF-α were purchased from R&D Systems ( Minneapolis, MN) and used at the final concentrations of 1 and 10 ng/ml, respectively, as previously reported (38, 40).

Cell proliferation

Cell proliferation was assessed using an [3H]thymidine uptake assay. [3H]thymidine (1 μCi) was added to 2 × 106 cells in 100 μl medium and incubated overnight at 37°C, 5% CO2. Cells were harvested and counted in a β-scintillation counter (TopCount; Packard, Downers Grove, IL).

MDM preparation and HIV-1 infection

Primary MDM were prepared as previously described (29). Briefly, PBMC from healthy donors undergoing leukopheresis were separated on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient. Suspension on 2 × 106 cells in DMEM supplemented with 10% heat-inactivated human serum, were purified by plastic adherence. After 2 h adherent cells were washed, and cultures were resuspended in DMEM containing human M-CSF (2 ng/ml; Sigma, Milan, Italy). After 24 h, cells were washed, treated with 10 mM PBS/EDTA for 1 min at 4°C, washed again, and scraped. Cells were then seeded into 24- or 6-well plastic plates ( Falcon; Becton Dickinson Labware, Lincoln Park, NJ) at 1 × 105 or 4 × 105 cells/well for standard infection and HIV DNA quantitation, respectively. Cells were allowed to differentiate for 5–7 days in the presence of M-CSF. After incubation with HIV at the multiplicity of infection of 0.1 for 2 h at 37°C in a humidified atmosphere with 5% CO2 in the presence or absence of PTX-B, cells were washed and cultivated in DMEM without M-CSF. Supernatants were collected every 2–3 days, replaced by fresh medium containing PTX-B, and stored at −80°C until tested for the presence of Mg2+-dependent reverse transcriptase (RT) activity (38, 40).

HIV-1 infection of U937 cells

U937 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium in the presence of 10% FCS before and after infection with the X4 HIV-1 clone. For infection, cells were seeded at 2 × 105 cells/ml in 96-well flat-bottom plates (Falcon) and infected with 1–2 × 105 virus particles diluted from a stock of pelleted virus (Advanced Biotechnology, Columbia, MD) at the multiplicity of infection of 1. After a 2-h infection at 37°C, cells were washed and resuspended in RPMI 1640 medium enriched with 10% FCS in the presence or absence of PTX-B.

Chronically HIV-infected U1 cell line

U1 cells were either left unstimulated or were stimulated with cytokines at the density of 2 × 106 cells/ml in RPMI 1640 (BioWhittaker, Walkerville, MD) containing 10% FCS. Cells were incubated with 1 μM PTX-B for 20 min at 37°C before the addition of cytokines. Viral expression was monitored by determination of Mg2+-dependent RT activity in culture supernatants.

HIV-1 strains

Two laboratory-adapted (ADA, Bal) and one primary (HIV92U660) R5 strains, and one laboratory-adapted X4 virus (LAI/IIIB) were used. All HIV stocks were derived from acute infection of primary PBMC stimulated with PHA (Sigma) for 72 h, washed, and resuspended at IL-2 (Boehringer Mannheim, Mannheim, Germany) (PHA blasts). At the peak of RT activity release, culture supernatants were passed through a 0.2-μm filter, aliquoted, and stored at −80°C. For HIV DNA quantitation, viral stocks were treated with 10 mM MgCl2 and 100 U/ml of RNase-free DNase (Boehringer Mannheim) for 1 h at room temperature before infection.

MDM infection with pseudotyped luciferase reporter viruses

Infection assays with luciferase readout was performed as previously described (41). Briefly, pseudotyped luciferase reporter viruses were produced by the Fugene 6 Transfection protocol (Boehringer Mannheim) in 293T cells. Cells were cotransfected with the env-deficient NL4–3 construct pNL-Luc and with a pSV vector expressing viral envelope glycoproteins derived from the amphotropic murine leukemia virus (MuLV) or the vesicular stomatitis virus (VSV) G protein (VSV-G). One million MDM were infected with 10 ng of p24 HIV-1 Gag Ag-pseudotyped virus in the presence or absence of PTX-B in a final volume of 100 μl. After 3 h of incubation at 37°C, 5% CO2, cells were resuspended in a final volume of 1 ml in the presence or absence of freshly added PTX-B. After 4 days, cells were washed once with PBS and lysed in 50 μl of 1 × reporter lysis buffer (Promega, Madison, WI). The luciferase activity was measured in terms of relative light units in a mixture of 100 μl of luciferase substrate (Promega) and 20 μl of cell lysate, using a MLX microplate luminometer (Dynex, Farmington Hills, MI).

HIV DNA quantitation by real-time PCR

The levels of HIV-1 DNA accumulation in MDM were determined by a TaqMan assay using an ABI 7700 Prism instrument (Applied Biosysystems, Foster City, CA) as previously described (42–44). MDM were washed and scraped in PBS/EDTA 0.02%, centrifuged at 12,000 rpm for 10 min, and harvested. Cell pellets were lysed in a buffer containing NaCl (0.3 M), Tris–HCl (0.01 M), urea (8 M), and SDS (10%). DNA was extracted by phenol–chloroform and ethanol precipitated. Fifty nanograms of DNA were amplified with the following primers derived from the R-U5 long terminal transcriptase (RT) primer l. After 3 h of incubation at 37°C, 5% CO2, cells were resuspended in a final volume of 1 ml in the presence or absence of freshly added PTX-B. After 4 days, cells were washed once with PBS and lysed in 50 μl of 1 × reporter lysis buffer (Promega, Madison, WI). The luciferase activity was measured in terms of relative light units in a mixture of 100 μl of luciferase substrate (Promega) and 20 μl of cell lysate, using a MLX microplate luminometer (Dynex, Farmington Hills, MI).
standard curve was obtained by serial dilutions of pNL 4-3 DNA with a linear distribution (r = 0.99) between 10 and 10^10 copies. Intersample variation was monitored by parallel Sybr green (Perkin-Elmer, Foster City, CA) PCR with an ABI 7700 Prism instrument (PE Biosystems, Warrington, U.K.) measuring total cellular DNA with primers specific for human GAPDH, sense primer 5'-ACCAGTCTCCAGCTCATCT-3', and antisense primer 5'-GGCCATCAGCCGACAGGTT-3'. The thermal cycling conditions were 50°C for 4 min, 95°C for 12 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min.

EMSA for NF-κB
Whole cell extracts were prepared in buffer C (20 mM HEPES, pH 8.0; 25% glycerol; 0.42 M NaCl; 1.5 mM MgCl2 ; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF) from 5 × 10^6 cells at 30 min and 4 h after stimulation in the presence or absence of PTX-B according to previously published procedure (46). The oligonucleotide probe for NF-κB 5'-GCTA CAAGGGACCTTCCGCTGGACGTTTCAGG was annealed to its complementary strand and labeled with [32P]ATP (Amersham, Little Chalfont, U.K.) using polynucleotide kinase (New England Biolabs, Beverly, MA). EMSA for detecting NF-κB DNA binding was performed as previously described (46). Briefly, equal amounts of cell extracts (10 μg) were added to a reaction mixture of 17 μl of buffer D (20 mM HEPES, pH 8.0; 25% glycerol; 100 mM KCl; 0.2 mM EDTA; 0.5 mM DTT; 0.5 mM PMSF) and 1 μl of poly(dI:dC) (5 mg/ml) (Pharmacia Upjohn, Piscataway, NJ). For supershift analysis, parallel aliquots of cell extracts were incubated in the absence or presence of 1 μg of polyclonal anti-p50 (N-terminal) or anti-p65 (N-terminal) Abs (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. One microliter of 10^6 cpm [32P]ATP-labeled double-stranded probe (0.5 ng) was then added, and the reaction was incubated at room temperature for 30 min and then run on a 5% acrylamide gel in 1× TBE (0.0045 M Tris-borate, 0.001 M EDTA). Radioactive bands were revealed by autoradiography of dried gels.

Quantification of HIV-1 RNA transcripts
Both unsupplied (US) and MS HIV-1 RNAs were quantified by competitive RT-PCR amplification of total cell-associated RNA from infected cells and known amounts of pMC-derived competitor RNA, as described (47). Quantification of DNase-treated RNA species was achieved by separate reactions. US transcripts were assayed by the SK38-SK39 primer pair generating two amplicons, wild-type (WT, 115 bp) and competitor (97 bp) RNA. MS messages were tested by the 659-413 MOD primer pair, generating two amplicons of 193 (WT) and 172 (competitor) bp, respectively. Different concentrations of competitor RNA (from 10^6 to 10^11 molecules in 2 μl) vs a constant amount of WT RNA, equivalent to 10^6 cells, were added to two separate series of reaction tubes in the presence of 100 U of Moloney MuLV RT (Life Technologies, Renfrewshire, Scotland), 25 pmol of antisense primers (either SK39 or 413 MOD), MgCl2 (1.5 mM), KCl (50 mM), Tris-HCl, pH 8.3 (10 mM), deoxyribonucleoside triphosphates (500 μM each), and 20 U of RNase inhibitor (Boehringer Mannheim) in a final volume of 20 μl. Reverse transcription was then performed at 42°C for 30 min, followed by a denaturation step at 95°C for 2 min. Thirty microliters of 1× PCR buffer containing 2 mM of MgCl2, 2.5 U of Taq Gold DNA polymerase (Perkin-Elmer) and 25 pmol of sense primer (either SK38 or 659) were then added, and samples were subjected to PCR amplification. The amplification profile was identical for reactions, one step at 95°C for 12 min, followed by 15 s at 95°C, 15 s at 60°C, and 60 s at 72°C for 50 cycles. The reactions for US and MS RNA were conducted in parallel using the same thermal cycler (Perkin-Elmer). After PCR, 25 μl of amplified products were loaded onto 4% NuSieve-agarose gels and electrophoresis was performed at 130 V for 60 min for both products. Competitive analyses were performed as previously described (41).

DEAE-dextran transfection of U1 and U937 cells
Five million cells were washed twice in PBS and transiently transfected with LTR-luciferase (Luc) encoding firefly luciferase under control of HIV-LTR, or with pRL-CMV DNA (CMV-Luc) encoding Renilla luciferase (2.5 μg in 2 ml of transfection buffer) using the hypotonic DEAE-dextran method (transfection buffer, 3.33 mM RPMI 1640, 125 μl 2 M Tris pH 7.5, 1.3 ml H2O, and 125 μl DEAE-dextran 10 mg/ml). Four hours after transfection, the cells were either left untreated or were treated with PTX-B for 20 min at 37°C, and either left unstimulated or were stimulated with TNF-α. After 48 h cells were lysed and analyzed by using the Luciferase Assay System (Promega) as suggested by the manufacturer.

Western blot analysis of cell-associated HIV-1 proteins
Cellular proteins were denatured by addition of an equal volume of 2× sample buffer (0.125 M Tris-HCl, pH 6.8; 4% SDS; 10% 2–2-ME; 20% glycerol) and heated for 5 min at 100°C before SDS-PAGE on a 12.5% gel and subsequent transfer to a nitrocellulose membrane (Hybond ECL; Amersham) by electroblotting. Membranes were blocked in 5% nonfat milk, 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.2% Tween 20 for 1 h at room temperature and further incubated overnight at 4°C with serum (1:1000 dilution) from an HIV-infected individual containing high titers of anti-HIV Abs. Ab binding was visualized by using HRP-conjugated anti-human Abs (Amersham Pharmacia Biotech, Piscataway, NJ). The signal was revealed by the ECL (Amersham) according to the manufacturer’s instructions.

Results
PTX-B inhibits HIV replication in acutely infected primary MDM and U937 cells
We have recently observed that PTX-B (Fig. 1) inhibited entry of R5 HIV-1 and replication of X4 viruses in activated PBMC (2). Therefore, we tested whether this molecule could also interfere with CCR5-dependent HIV-1 replication in primary MDM. Indeed, inhibition of viral replication was observed when MDM cultures were infected in the presence of 1–10 nM of PTX-B (IC50 ~0.5 nM, Fig. 2A). In addition, PTX-B strongly inhibited X4 viral replication in U937 cells (data not shown), consistent with our previous observation in acutely infected primary T lymphocytes (2). No cytoxicity was observed in macrophages or U937 cells treated with PTX-B, as evidenced by trypan blue exclusion and MTT assays (data not shown).

In some experiments MDM were treated with 10 μM of the RT inhibitor AZT after 12 days of infection to prevent further HIV-1 spreading, as described (48, 49). In parallel, cells were also treated with either PTX-B alone or in combination with AZT. Both AZT and PTX-B partially curtailed the production of new virions, measured by supernatant-associated RT activity (50). Because this concentration of AZT completely prevents de novo HIV infection of MDM (data not shown) the fraction of RT activity still produced

FIGURE 1. Purity of PTX-B. Aliquots (3 μg) of A-protomer, PTX-B, and PTX were fractionated by SDS-PAGE on a 15% gel and revealed by silver staining. PTX subunits are indicated on the right; m.w. markers are indicated on the left.
PTX-B inhibits HIV-1 replication in primary MDM cultures. A. Concentration-dependent effect of PTX-B. MDM cultures were pretreated for 20 min with the indicated concentrations of PTX-B and then infected with the R5 HIV-1 strain ADA (5000 cpm of RT activity per 10⁶ cells). Two hours after adsorption, the viral inoculum was diluted and then removed. Fifty percent of the medium was exchanged every 72 h throughout the culture, with the indicated concentrations of PTX-B being readded together with fresh medium. RT activity in culture supernatants was measured at indicated times; the results are presented as mean of duplicate cultures indicated concentrations of PTX-B being readded together with fresh medium. RT activity in culture supernatants was measured at indicated times; the results are presented as mean of duplicate cultures ± SD. B, PTX-B inhibitory effect on established MDM infection. MDM were left untreated or pretreated for 20 min at 37°C with PTX-B and inoculated with R5 HIV-1,μLV. After 12 days (arrow), aliquots of infected MDM that were not earlier incubated with PTX-B (+) were treated with AZT (10 µM), PTX-B, or AZT plus PTX-B.

PTX-B inhibits TNF-α-dependent expression of HIV independently from activation of NF-κB

To investigate the postintegration inhibitory effect of PTX-B, chronically infected U1 cells were stimulated with TNF-α in the presence or absence of PTX-B. TNF-α is a classic transcriptional inducer of viral expression mainly operating via activation of the cellular transcription factor NF-κB (35, 36, 53, 54). In this regard, it has been independently reported that PTX, but not PTX-B, blocked TNF-α-induced HIV-1 expression in U1 cells through inactivation of Gs protein (55). However, in contrast to that study, we observed that PTX-B partially inhibited TNF-α-induced HIV-1 expression in U1 cells in a concentration-dependent manner, with a 50% inhibitory effect on virus production observed at concentrations as low as 40 pM (Fig. 4). No decrease in cell viability as determined by the trypan blue dye exclusion test (data not shown) or cell proliferation (measured by [3H]thymidine uptake) was observed in PTX-B-treated vs untreated cells in the presence or absence of TNF-α (data not shown). As expected, a decrease of both US and, particularly, MS transcripts was observed in PTX-B-treated U1 cells stimulated with TNF-α, compared with untreated and stimulated cells (Fig. 4B).

To further characterize the effect of PTX-B on LTR-driven transcription, U1 cells were transiently transfected with a prototypic HIV-1 LTR-luciferase reporter plasmid and were either left unstimulated or were stimulated with TNF-α. PTX-B significantly decreased both the basal and the cytokine-stimulated luciferase activities in transfected U1 cells (Fig. 4C). Similar findings were noted when U1 cells were transfected with LTR-luciferase constructs derived from different viral clades (56) (provided by B. Berkhout, University of Amsterdam, Amsterdam, The Netherlands) or when uninfected U937 cells, instead of the U1 cell line, were transfected with a LTR-luciferase reporter plasmid. As observed in LTR-luciferase reporter assay, PTX-B exerted a profound inhibitory effect on the synthesis of TNF-α-induced HIV DNA in MDM infected with HIV-1,μLV, as determined by quantitative real-time PCR (Fig. 3B). Taken together, these results indicate that the anti-HIV activity of PTX-B is not restricted to CD4+ T lymphocytes, but extends to mononuclear phagocytes.
were used (data not shown). Therefore, the decreased levels of luciferase activity observed in TNF-α-stimulated cells in the presence of PTX-B most likely reflects a down-regulation of basal rather than cytokine-induced transcription. In support of this interpretation, PTX-B did not interfere with TNF-α-induced activation of NF-κB, as observed by EMSA 30 min and 4 h after stimulation (Fig. 5). In this regard, U1 cells resemble “minus” U937 cell clones that generate upon activation an heterodimeric complex composed of p50 and a truncated p65 (p65Δ) subunits, a complex that is indistinguishable by banding pattern from that composed of p50 homodimers (46). Therefore, supershift analysis mediated by Abs specific for these subunits was performed in both unstimulated and TNF-α-stimulated U1 cells in the presence or absence of PTX-B. As shown in Fig. 5, no qualitative or quantitative differences in the activation or composition of NF-κB complexes were observed in U1 cells that have been treated or not with PTX-B. Altogether, these results indicate that PTX-B inhibits viral transcription without affecting the capacity of TNF-α to activate NF-κB.

PTX-B suppresses HIV-1 expression in U1 cells stimulated with IL-6

In addition to TNF-α, IL-6 up-regulates HIV expression in U1 cells as well as virus replication in primary MDM and IL-2-stimulated PBMC (38, 57, 58). Unlike TNF-α, IL-6 activates HIV expression in U1 cells predominantly at a posttranscriptional level as determined by combined Northern blot and nuclear run-on analyses (38). PTX-B strongly inhibited virus expression in U1 cells stimulated with IL-6 (Fig. 6A) without any effect on cell viability or proliferation (data not shown). By using a sensitive RT-PCR technique, ~10- to 100-fold fewer US and MS viral transcripts were detected in U1 cells stimulated with IL-6 in comparison to cells incubated with TNF-α, respectively (Fig. 6B vs 4B), although the differences in RT activity were contained within 2- to 3-fold. PTX-B strongly inhibited HIV US transcripts with a relative increase of MS messages (Fig. 6B); consistently, PTX-B also inhibited the synthesis of cell-associated viral proteins (Fig. 6C).

PTX-9K/129G inhibits acute HIV replication in activated T cells and MDM, and virus expression in stimulated U1 cells

PTX-9K/129G, a modified molecule successfully used in protocols of vaccination against Bordetella pertussis infection in humans (59), lacks the A-protomer-specific enzymatic activity but retains a functional B subunit (16). PHA blasts were infected with either
HIV-1 LAI/IIIB or with the primary R5 strain HIV2US660 in the presence or absence of different concentrations of PTX, PT-9K/129G, or PTX-B. Unlike PTX-B and PT-9K/129G, the holotoxin PTX demonstrated an inhibitory activity that did not titrate despite the broad range of concentrations tested (ranging from $10^{-18}$ to $10^{-14}$ M) (Fig. 7A). When MDM were infected with HIV-1 BaL in the presence or absence of PTX, PTX-B, or PT-9K/129G, a similar profile of inhibition of virus replication was observed (Fig. 7B). Finally, PT-9K/129G and PTX-B showed superimposable concentration-dependent HIV inhibitory activity in TNF-α-stimulated U1 cells, whereas PTX suppressive effect remained constant at all the concentrations tested (Fig. 7C), as previously reported (55), thus indicating additional antiviral activities associated most likely with the A-protomer.

**Discussion**

In this study we have demonstrated that the binding subunit of PTX inhibited R5 virus multiplication in primary human MDM and X4 HIV-1 replication in acutely infected promonocytic U937 cells. The effect observed in MDM correlated in part with R5 virus entry inhibition, as previously reported for T lymphocytes (2). In addition, several lines of experimental evidence presented here and in a separate report (39) indicate a broader inhibitory effect of PTX-B on postentry events of the virus life cycle during acute infection. PTX-B inhibited HIV-1 expression in promonocytic U1 cells chronically infected with HIV and stimulated with TNF-α or IL-6. Of interest, the down-regulation of TNF-α-induced HIV expression did not affect the activation of NF-κB. Finally, PT-9K/129G showed an anti-HIV profile superimposable to that of PTX-B.

Mononuclear phagocytes are known to play a crucial role in HIV infection both as viral reservoirs and as sources of replicating HIV, particularly in organs such as the brain and the lung (25). They represent a major obstacle for eradicating HIV-1 infection due to their long lifespan and their relative resistance to cytopathic effects of HIV infection (27, 28). AZT is known to block HIV spreading in both macrophages and T lymphocytes (60) but is devoid of antiretroviral activity in cells containing integrated proviruses, such as chronically infected cell lines (61). In this study we have compared AZT and PTX-B for their in vitro efficacy once added to an ongoing infection of MDM. Of interest, PTX-B demonstrated a superior capacity of inhibiting viral production compared with AZT, whereas no synergistic or additive effects were observed when the two agents were added together. We interpret these results as evidence in support of a broader spectrum of anti-HIV effects induced by PTX-B in comparison to AZT.

TNF-α and IL-6 were previously characterized as prototypic transcriptional and posttranscriptional activators of HIV-1 expression, respectively, in cells carrying integrated proviruses (36–38, 40). PTX-B significantly reduced the level of MS and US viral RNAs induced by TNF-α, along with suppressive effects on viral protein expression and output of the virion progeny as measured by RT activity in culture supernatants. In this regard, it is well established that TNF-α-mediated effects are mostly dependent upon the activation of the cellular transcription factor NF-κB (36, 37, 53, 54). Quite surprisingly, PTX-B did not perturb the ability of TNF-α to activate NF-κB in U1 cells both early (30 min) and up to 4 h after cytokine stimulation, although it inhibited the basal LTR-mediated transcription in both U1 and U937 promonocytic...
dichotomous effect of PTX-B on MS and US HIV-1 RNAs in IL-6-stimulated U1 cells suggests that PTX-B may interfere with some element of the Rev/RRE posttranscriptional axis fundamental for HIV protein expression (reviewed in Ref. 64).

Together with our previous reports (2, 39), this study demonstrates that PTX-B can interfere with both R5 and X4 HIV-1 infection of CD4+ T lymphocytes and macrophages by at least two distinct mechanisms: a blockade of R5 HIV-1 entry through desensitization of CCR5, and an inhibitory effect on viral protein synthesis through inhibition of LTR-mediated transcription independently from the activation of NF-κB. Thus, PTX-B is a nontoxic molecule that could be tested for its potential efficacy in preventing or reducing HIV-1 multiplication in exposed or infected individuals. This possibility is strongly supported by the fact that PT-9K/129G, here shown to possess anti-HIV activity superimposable to that of PTX-B, has been proven to be safe (59) and was approved for human use as a component of the vaccine against Bordetella pertussis infection (18).

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Unlike TNF-α, IL-6 up-regulates protein and virion expression in U1 cells mostly at posttranscriptional levels (37). Indeed, 10- to 100-fold lower levels of viral transcripts were induced by IL-6 in U1 cells in comparison to those activated by TNF-α, as measured by a sensitive quantitative PCR analysis. PTX-B suppressed accumulation of IL-6-induced HIV-1 RNA and proteins. In contrast to TNF-α-stimulated cells, where both classes of HIV-1 RNAs were down-regulated by PTX-B, the levels of IL-6-induced MS HIV-1 RNAs were actually increased by incubation with PTX-B. This


