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Dual Role of Prostratin in Inhibition of Infection and Reactivation of Human Immunodeficiency Virus from Latency in Primary Blood Lymphocytes and Lymphoid Tissue

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To design strategies to purge latent reservoirs of human immunodeficiency virus type 1 (HIV-1), we investigated mechanisms by which a non-tumor-promoting phorbol ester, prostratin, inhibits infection of CD4$^+$ T lymphocytes and at the same time reactivates virus from latency. CD4$^+$ T lymphocytes from primary blood mononuclear cells (PBMC) and in blocks of human lymphoid tissue were stimulated with prostratin and infected with HIV-1 to investigate the effects of prostratin on cellular susceptibility to the virus. The capacity of prostratin to reactivate HIV from latency was tested in CD4$^+$ T cells harboring preintegrated and integrated latent provirus. Prostratin stimulated CD4$^+$ T cells in an aberrant way. It induced expression of the activation markers CD25 and CD69 but inhibited cell cycling. HIV-1 uptake was reduced in prostratin-stimulated CD4$^+$ T PBMC and tissues in a manner consistent with a downregulation of CD4 and CXCR4 receptors in these systems. At the postentry level, prostratin inhibited completion of reverse transcription of the viral genome in lymphoid tissue. However, prostratin facilitated integration of the reverse-transcribed HIV-1 genome in nondividing CD4$^+$ T cells and facilitated expression of already integrated HIV-1, including latent forms. Thus, while stimulation with prostratin restricts susceptibility of primary resting CD4$^+$ T cells to HIV infection at the virus cell-entry level and at the reverse transcription level, it efficiently reactivates HIV-1 from pre- and postintegration latency in resting CD4$^+$ T cells.

The persistence of human immunodeficiency virus type 1 (HIV-1) within infected individuals constitutes a major obstacle to the control of HIV-1 infection. Although highly active antiretroviral therapy (HAART) has been successful in reducing HIV-1 plasma viremia to undetectable levels in a substantial proportion of treated patients, replication-competent HIV persists in resting memory CD4$^+$ T cells (10, 14, 48). Also, HIV-1 persists in various compartments inaccessible to present HIV-1 therapy (6). In resting memory CD4$^+$ T cells, HIV-1 persists in a latent form (5, 8, 10, 13) or replicates residually (16, 38, 51, 52). Residual replication is responsible for most of the viral rebound that follows the cessation of HAART (12, 16). Furthermore, latent infection seems to provide a mechanism for lifelong persistence of HIV-1 (13). The viral latency in the reservoir of resting memory CD4$^+$ T cells is characterized by the integration of HIV-1 provirus within the host cell genome and by the absence of nonspliced HIV-1 RNA and of virus production (5, 10, 16). In contrast to this "postintegration latency," infection of resting memory CD4$^+$ T cells without minimal cell stimulation results in "preintegration latency," characterized by the presence of nonintegrated viral DNA in the cell nucleus. The nonintegrated viral DNA is cleared with a half-life of about 1 day (3, 28, 33, 38, 39, 46, 50) but can be rescued upon cell activation. It has been suggested that activation of latently infected T cells in the presence of HAART shortens the half-life of the HIV reservoir because, presumably, reactivation of latent (integrated) HIV is followed by host cell death (7, 12, 47).

Here, we sought to investigate reactivation of HIV from pre- and postintegration latency in resting memory T cells by the presence of prostratin (12-deoxyphorbol-13-acetate) (43–45). Prostratin is an activator of protein kinase C and a potent antitumor agent that directly blocks tumor promotion by phorbol-12-myristate-13-acetate (TPA). Prostratin was originally extracted from the Samoan medicinal plant *Homalanthus nutans* and has been described as a drug that inhibits HIV replication and cell killing in vitro (24, 25, 30). Unlike other phorbol esters (31), prostratin activates cells (e.g., upregulates expression of CD25 and CD69) without induction of cell cycling (29, 30) and exhibits a cytostatic effect on T-cell lines (24). Prostratin has previously been reported to activate viral expression in latently infected cell lines (24, 25), in peripheral blood mononuclear cells (PBMC) of infected patients (30), and in HIV-infected SCID-hu (Thy/Liv) mice (2, 29). Whereas previous studies have predominately described effects of the presence of prostratin on isolated cells (cell lines and PBMC) infected with HIV, the critical events in HIV infection in vivo occur in lymphoid tissue. Therefore, in the present work we studied the role of prostratin in HIV replication in lymphoid tissue ex vivo (17–20, 23, 32). Our results show that prostratin may be a promising drug which, in the context of human lymphoid tissue cytoarchitecture, inhibits infection of CD4$^+$ T

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lymphocytes and at the same time reactivates virus from latency.

MATERIALS AND METHODS

Preparation of HIV-1-infected resting memory T cells derived from PBMC. HIV-infected resting T cells generated in vitro were prepared as previously described in detail (20). Briefly, PBMC of healthy donors were separated on Ficoll-Hypaque gradients. Aliquots of 2×10^8 PBMC, depleted of monocytes by adherence to plastic, were activated with phytohemagglutinin-P (PHA) (Difco, Franklin Lakes, N.J.) at 2 μg/ml in RPMI 1640 supplemented with 15% fetal calf serum (FCS) and antibiotics for 3 days. After cell-clump disintegration 6 days from the beginning of activation, peripheral blood lymphocytes suspended at 2×10^7 cells per milliliter were treated for 1 h with anti-CD8 antibody at the saturating concentration at 4°C. The cell suspension was incubated with magnetic beads coated with goat anti-mouse antibody (Miltenyi Biotech, Bergisch Gladbach, Germany), and the positively labeled cells were removed as recommended by the manufacturer. $CD4^+$ T cells were then infected with HIV-1 NL4-3 (1). Alternatively, $CD4^+$ T cells were transduced with an HIV-1-derived vector (HDV; kindly provided by D. R. Littman, Skirball Institute of Biomolecular Medicine, New York, N.Y.) that was prepared and used as described by Unutmaz et al. (46). Approximately 3 weeks after activation with PHA, the residually activated T cells cultivated in RPMI medium supplemented with 200 U of recombinant interleukin-2 (IL-2; Chiron)/ml were removed from the cell culture by incubation with monoclonal antibody against CD25, CD69, and HLA-DR followed by magnetic bead separation.

Human tonsil tissue culture. Ex vivo-infected human lymphoid tissue supports productive infection with HIV-1 virus without exogenous activation. Tonsillar tissue blocks placed on collagen sponge gels were infected with the X4 virus HIV-1 NL4-3 or with HDV as described elsewhere (17, 21). In a typical experiment, 3 to 5 μl of clarified virus-containing medium—approximately 150 50% tissue culture infective doses ($TCID_{50}$) per block—was applied to the top of each tissue block. In some experiments, tonsillar tissue was activated with PHA (2 μg/ml) or prostratin (10 μM) 2 days before infection. Productive infection was assessed by measurement of p24gag levels accumulated in the culture medium during the 3 days between successive medium changes. Flow cytometry analysis was performed on cells mechanically isolated from control and ex vivo-infected blocks of human lymphoid tissue. Lymphocytes were identified according to their light-scattering properties and then analyzed for the expression of lymphocyte and activation markers.

Immunofluorescence analysis. For analysis of cell surface marker expression, 2×10^5 cells were washed in phosphate-buffered saline containing 0.5% FCS and 0.02% sodium azide and incubated for 20 min at the ambient temperature in the presence of the appropriate antibodies at a twofold saturating concentration. CD3, CD4, CD25, HLA-DR, and CD69 antibodies conjugated with different chromophores (fluorescein isothiocyanate, phycoerythrin, TriColor-conjugated antibody, peridinin chlorophyll protein, and allophycocyanin) were used for staining (all were purchased from Pharmingen Inc., San Diego, Calif.). For immunodetection of intracellular expression of HIV-1 p24gag, the cells were fixed and permeabilized with Cytofix-Cytoperm (Pharmingen) and stained with the anti-p24 antibody KC57 RD1 (Coulter, Miami, Fla.). Cells were then washed, fixed in 1% formaldehyde, and analyzed with FACScalibur and CellQuest software (Becton-Dickinson, Le Pont de Claix, France).

Cellular proliferation. PBMC were stimulated with PHA for 2 days before addition of 10 μM prostratin. Cellular proliferation was assessed 1 day after costimulation with PHA and prostratin by measurement of DNA synthesis. A total of 10^5 cells were incubated with [3H]thymidine (10 μCi/ml) and harvested onto glass filters. Thymidine incorporation into DNA was measured with a liquid scintillation counter. The relative proliferation index was calculated as the ratio of PHA- and prostratin-treated samples to controls treated only with PHA.

Cell cycle analysis. Cycling cells were detected by means of DNA labeling with 7-amino-actinomycine D (7AAD) (20). Briefly, 5×10^5 cells were incubated with 20 μM 7AAD for 30 min at room temperature in the presence of 0.004% saponin. For flow cytometric analysis, cells were gated by means of forward and side scatters. Resting PBMC and activated peripheral blood lymphocytes were used as standards.

Virus cell-entry analysis. To measure the HIV-1 uptake, 3×10^6 $CD4^+$ T cells treated or not treated with prostratin were exposed to HIV-1 for 4 h at 37 or 4°C, digested with trypsin XIII for 5 min, and lysed in a 0.2-ml volume of TNE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) with 0.05% Triton X-100. Viral entry was evaluated by quantification by enzyme-linked immunosorbent assay of the difference in intracellular p24gag levels in the cell lysate at both temperatures.

Real-time PCR. DNA for PCR analysis was prepared with a DNeasy tissue kit from QIAGEN SA (Courtaboeuf, France). Real-time PCR was performed to quantify initiated and completed reverse transcripts. The early primer set MH535 (forward) (4) and U5As (5′-GCTAGAGATTTTCCACACTGAC-3′) (reverse) and the late primer set MH531 (forward) and MH532 (reverse) (4) were used to detect early and late reverse transcription products, respectively. We performed amplification and detection on an Applied Biosystems Prism 7000 sequence detection system using a Taqman universal master mix (Perkin-Elmer–Applied Biosystems, Foster City, Calif.), 300 nM forward primer, 300 nM reverse primer, 100 nM probe, and 100 to 500 ng of template DNA in a 25-μl reaction volume (22). After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out, each cycle consisting of 15 s at 95°C followed by 1 min at 60°C. We analyzed the reactions by use of an ABI Prism 7000 sequence detection system (Perkin-Elmer–Applied Biosystems).

HIV-1 integration assay. The Alu-HIV-1 DNA junctions present in PBMC of infected individuals were amplified by primers Alu (sense) and L1 (antisense) (49); the amplification products were subjected to a second round of PCR with HIV-1 long terminal repeat (LTR)-specific primers L2 and L3 (49). PCR products were visualized after agarose gel electrophoresis with ethidium bromide. 8E5 cells used as a positive control were obtained through the AIDS Research and Reference Reagent Program from T. Folks (15).

RESULTS

Prostratin stimulates expression of CD25 and CD69 in PBMC but without stimulation of cell cycling. We investigated the induction of activation markers and proliferation on resting $CD4^+$ T cells that were depleted of monocytes and $CD8^+$ and $CD25^+$ lymphocytes; these $CD4^+$ T cells were from PBMC stimulated either with prostratin or with PHA and infected with HIV-1 on day 3 after stimulation. The analyses were performed by means of flow cytometry on days 1, 3, and 7 after stimulation (Fig. 1A). Although CD25 was expressed in up to 10% of nonstimulated $CD4^+$ T cells, its expression in these cells reached less than 1.2% of the maximal CD25 level (Fig. 1B to D). In consistency with previously published results (29), prostratin stimulated expression of activation markers on the surface of CD4 T cells. There were approximately 3 times more $CD25^+$ $CD4^+$ T cells and 40 times more $CD69^+$ $CD4^+$ T cells in cultures treated with 10 μM prostratin than in untreated controls (Fig. 1B to F). In contrast to prostratin results, the polyclonal mitogenic activator PHA was more efficient at inducing the expression of CD25 than the expression of CD69 (a 7.5-fold increase for CD25 versus a 4.0-fold increase for CD69 compared with the results seen with the untreated control). Full stimulation of CD25 was reached with 10 μM prostratin (Fig. 1C). The expression of CD25 increased gradually over time throughout the experiment. In contrast, CD69 expression induced by prostratin stimulation as well as CD25 expression induced by PHA stimulation reached a maximum at day 3 poststimulation and then dropped (Fig. 1D and E). We found that the presence of prostratin at a concentration of 10 μM inhibited the proliferation of PBMC activated for 2 days with PHA (Fig. 1H). The results shown in Fig. 1H demonstrate that this inhibition occurred in a dose-dependent manner ($n = 4$; $r^2 = 0.66$ [$P = 0.024$]). Thus, on the one hand prostratin induced strong cell surface expression of the T-cell activation markers CD25 and CD69, and on the other hand it inhibited mitogen-triggered T-cell proliferation.

Prostratin downregulates CD4 and CXCR4 in PBMC. In PBMC treated for 3 days with prostratin, CD4 and CXCR4 were expressed by fewer cells and at lower levels than in untreated control PBMC (Fig. 1G). Whereas there was a 40% decrease of CD4-expressing cells and a 54% decrease of

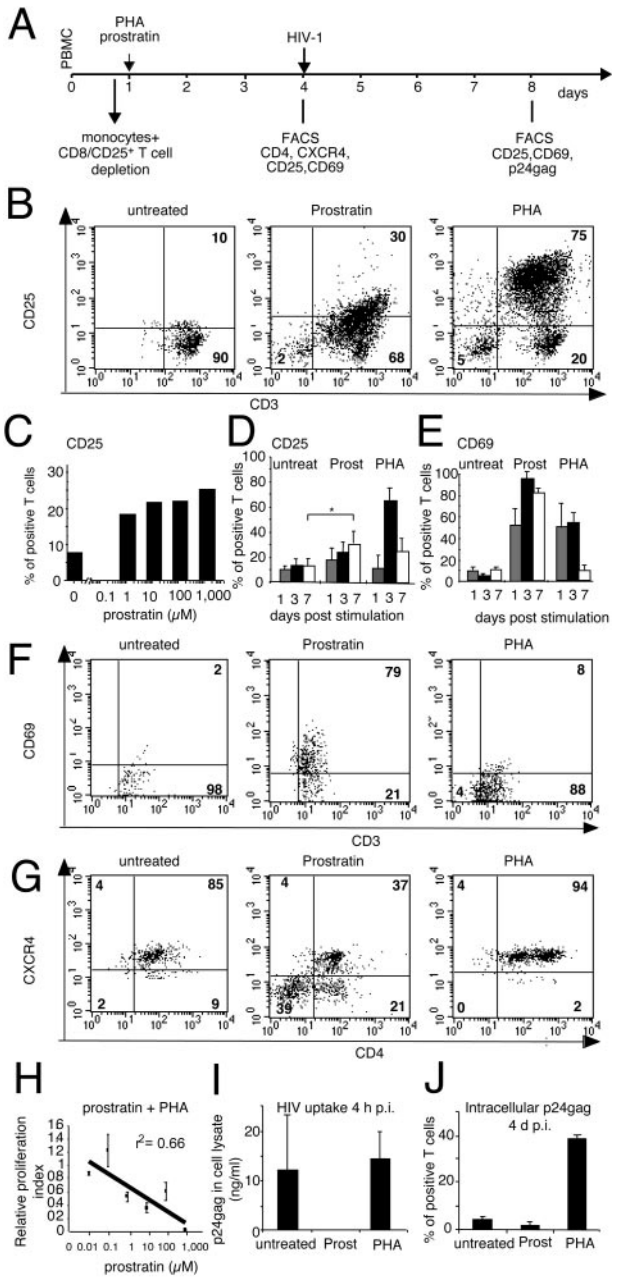


FIG. 1. HIV-1 infection of prostratin-treated PBMC. PBMC depleted of monocytes by overnight adherence to plastic and depleted of $CD8^+$ and of $CD25^+/CD69^+/HLA\text{-}DR^+$ cells by monoclonal antibody were stimulated either with 10 μM prostratin (Prost) or with PHA (2 μg/ml) and infected with HIV-1 NL4-3 3 days poststimulation. (A) Time schedule of cell culture and HIV-1 challenge. The fluorescence-activated cell sorter and cell proliferation analyses whose results are shown in panels B, C, F, G, and H were performed at 3 days poststimulation. FACS, fluorescence-activated cell sorter. (B to F) Induction of CD25 (B, C, and D) and of CD69 (E and F). (D to E) Kinetics of CD25 (D) and CD69 (E) expression in prostratin- and PHA-stimulated cultures. The asterisk denotes statistical significance ($P < 0.02$). untreat, untreated. (G) The effect of prostratin on the expression of CD4 and CXCR4. (H) The effect of prostratin on the relative proliferation index in PBMC activated with PHA (2 μg/ml) ($n = 4$). The relative proliferation index was calculated as the ratio of incorporation of [^{3}H]thymidine into PHA- and prostratin-treated samples to that in controls treated only with PHA. (I to J) The effects of prostratin on the cellular uptake of HIV-1 (I) and on the number of productively infected cells (J). To measure the HIV-1 uptake, cells treated or not treated with prostratin were exposed to HIV-1 for 4 h at 37 or 4°C, and viral entry was evaluated by quantification by enzyme-linked immunosorbent assay of the difference in intracellular p24gag levels at these temperatures. To measure the amounts of productive infection, cells treated or not treated with prostratin were exposed to HIV-1 for 4 days, and the numbers of infected cells were evaluated by means of flow cytometry after staining for intracellular p24gag. Typical dual-parameter plots from three experiments with indistinguishable results are shown in panels B, F, and G. Numbers displayed in each quadrant in these panels are percentages of positive cells. The results shown are the means of eight different experiments with PBMC from three donors.

CXCR4-expressing cells, the level of CD4 cell surface expression was reduced by 88% (the mean fluorescence intensity of CD4 dropped from 90 arbitrary units in the untreated control to 11 arbitrary units in prostratin-treated cells), in consistency with previously published results (24, 25, 30). The reduction of expression of these receptors was already evident 12 h after activation and remained evident on day 7 after activation (data not shown). In contrast, expression of CD4 and CXCR4 receptors was only slightly changed by mitogenic activation with PHA (there were increases of 2% for CD4-expressing cells and 10% for CXCR4-expressing cells compared with untreated controls) (Fig. 1G). Thus, unlike PHA, prostratin downregulates the HIV-1 receptors CD4 and CXCR4 in peripheral T cells.

Prostratin inhibits HIV-1 cell entry and replication. We estimated the HIV-1 uptake in prostratin-treated resting $CD4^+$ T cells characterized previously for activation and proliferation status and for the expression of HIV-1 receptors. HIV-1 uptake was assessed on the basis of different levels of internalized p24gag in the lysates of $CD4^+$ T cells held at 37 and 4°C 4 h postinfection, after cell surface-adsorbed p24gag had been removed by trypsin digestion (Fig. 1I). The levels of HIV-1 entry into cells pretreated with prostratin were lower (0.4 ± 0.3 ng of p24gag per ml of cell lysate) than in untreated control cells (12.0 ± 11 ng of p24gag per ml of cell lysate; $n = 4$). Prostratin-inhibited HIV-1 cell entry is consistent with the downregulation of both CD4 and CXCR4. In agreement with these results, productive infection with HIV-1 was not detected in resting $CD4^+$ T cells treated with prostratin and inoculated with the virus (Fig. 1J).

Prostratin reactivates HIV-1 from preintegration latency in resting $CD4^+$ T cells. To investigate the effect of the presence of prostratin on HIV-1 infection in cells harboring nonintegrated viral genomes, we purified resting $CD4^+$ T cells from monocyte-depleted PBMC by means of negative immunoselection with anti-CD8/CD25/CD69/HLA-DR antibodies. The composition of the resulting population, as evaluated with flow cytometry, was 98.5% resting CD4 T cells. These cells were inoculated with HIV-1 NL4-3 (Fig. 2A), and 1 day later they were activated with prostratin. We verified the lack of integrated proviruses in resting $CD4^+$ T cells in the absence of prostratin stimulation by means of Alu PCR of limiting dilutions of genomic DNA extracted 3 days after HIV-1 infection (Fig. 2B). The end point dilution at which a positive Alu PCR signal was detected in resting $CD4^+$ T cells (1-μg aliquot) contained 1,000 times more DNA than that of the control

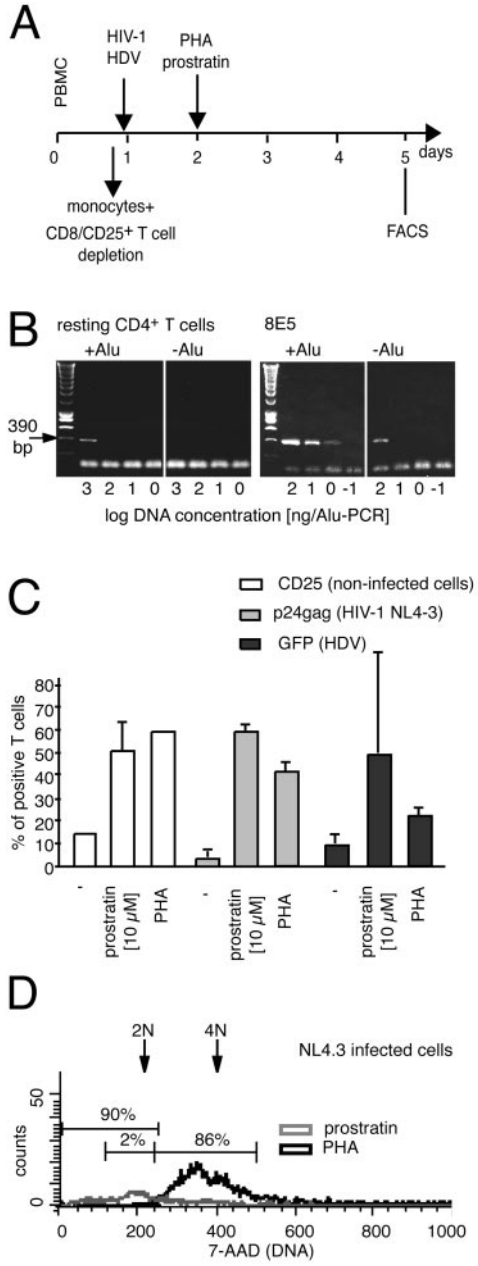


FIG. 2. Effect of prostratin on resting $CD4^+$ T cells infected with HIV-1. PBMC were depleted of monocytes, $CD8^+$ cells, and $CD25^+$/$CD69^+$/$HLA\text{-}DR^+$ cells and were inoculated with HIV-1 (approximately 1 $TCID_{50}$ per cell) or HDV (approximately 5 $TCID_{50}$ per cell) on day 1 of culture. These cells were reactivated with 10 μM prostratin, with PHA (2 μg/ml), or with soluble anti-CD3/CD28 monoclonal antibodies (1 μg/ml) in the presence of 10 μM AZT and 5 μM nevirapine on day 1 postinfection. (A) Time schedule of the experiments. FACS, fluorescence-activated cell sorter. (B) Alu PCR of 10-fold serial dilutions of genomic DNA extracted from resting treated $CD4^+$ T cells inoculated with HIV-1 and from control cell line 8E5. Nested PCR amplifications were conducted with the primer pairs as follows: a first round with primer pair Alu and L1 (L1 is an antisense primer localized in LTR) (+Alu) and a control round in which the forward Alu primer was omitted (−Alu) were followed by a second round of PCR with HIV-1-specific primers from the U3 part of the HIV-1 LTR. Amplification products were run on agarose gels and visualized by means of ethidium bromide staining. (C) Percentages of cells positive for CD25 (open bars), p24gag (light-gray-shaded bars), and GFP (black bars); means of six different experiments on PBMC from three different donors are shown. (D) Effect of prostratin on cell cycling of resting $CD4^+$ T cells infected with HIV-1 NL4-3. DNA content was determined by staining with 7ADD. Nonactivated or PHA-activated uninfected PBMC depleted of monocytes and $CD8^+$ cells were used as standards for 2 and 4 N DNA content. Horizontal bars aligned with the labels of untreated, prostratin-treated, and PHA-treated cells indicate the limits of the areas of 2 and 4 N cells.

genomic DNA extracted from T-cell line 8E5 (1-ng aliquot), which harbors one integrated provirus per cellular genome (15). Thus, in the absence of prostratin stimulation, resting $CD4^+$ T cells inoculated with HIV-1 NL4-3 harbored predominantly nonintegrated provirus.

In parallel, we inoculated these cells with an HDV (a replication-incompetent and noncytopathic vector lacking *vif*, *vpr*, *vpu*, and *env* and with an insertion of green fluorescent protein [GFP] in the *nef* open reading frame, pseudotyped with the vesicular stomatitis virus [VSV] glycoprotein G). Expression of HDV was evaluated by measurement of the fluorescence of GFP. To prevent HIV-1 secondary infection cycles after prostratin or PHA stimulation, cells were treated with 10 μM zidovudine (AZT) and 5 μM nevirapine. HDV-inoculated cells did not require this procedure, since this virus is replication incompetent. A flow cytometric analysis performed at 3 days after infection showed that among nonstimulated resting $CD4^+$ T cells exposed to HIV-1 NL4-3, 3% ± 1% ($n = 7$) were positive for p24gag, whereas among cells exposed to HDV, 8% ± 2% ($n = 5$) were positive for GFP (Fig. 2C). However, after stimulation with 10 μM prostratin, 60% ± 2% of HIV-1-exposed cells became p24gag positive and 50% ± 40% of HDV-inoculated cells expressed GFP ($n = 4$). In the control PHA-stimulated culture, 42% ± 4% of HIV-1-exposed cells became p24gag positive and 23% ± 2% of HDV-inoculated cells expressed GFP ($n = 4$).

To confirm that the productive HIV-1 infection in prostratin-stimulated $CD4^+$ T lymphocytes occurs in resting T cells, we investigated the expression of p24gag in cells with a DNA content of 2 or 4 N as measured with 7AAD (Fig. 2D). The majority (90%) of HIV-1-infected T cells that expressed intracellular p24gag in prostratin-stimulated cultures had a DNA content of 2 N. In contrast to results seen with cultures stimulated with prostratin, in PHA-stimulated cultures 86% of $p24gag^+$ T cells were found among cells with a DNA content of 2 N to 4 N. Thus, in contrast to the PHA-mediated increase, the prostratin-mediated increase in the number of $p24gag^+$ cells was not due to the stimulation of cell division but rather was caused by a release of HIV-1 from preintegration latency.

Prostratin reactivates HIV-1 from postintegration latency. To generate resting $CD4^+$ T cells harboring integrated latent proviruses, we inoculated PHA-activated PBMC with HIV-1 NL4-3 or with HDV. After the removal of activator, these cells return to the G_0/G_1 phase. The residually activated cells were immunodepleted with anti-CD25/CD69/HLA-DR antibodies (Fig. 3A). As a result, we obtained resting memory $CD4^+$ T cells that harbored the HIV-1 genome (20, 34). We stimulated these cells with prostratin, TPA, or anti-CD3/CD28 antibodies in the presence of 10 μM AZT and 5 μM nevirapine.

We assessed the proportion of resting $CD4^+$ T cells bearing

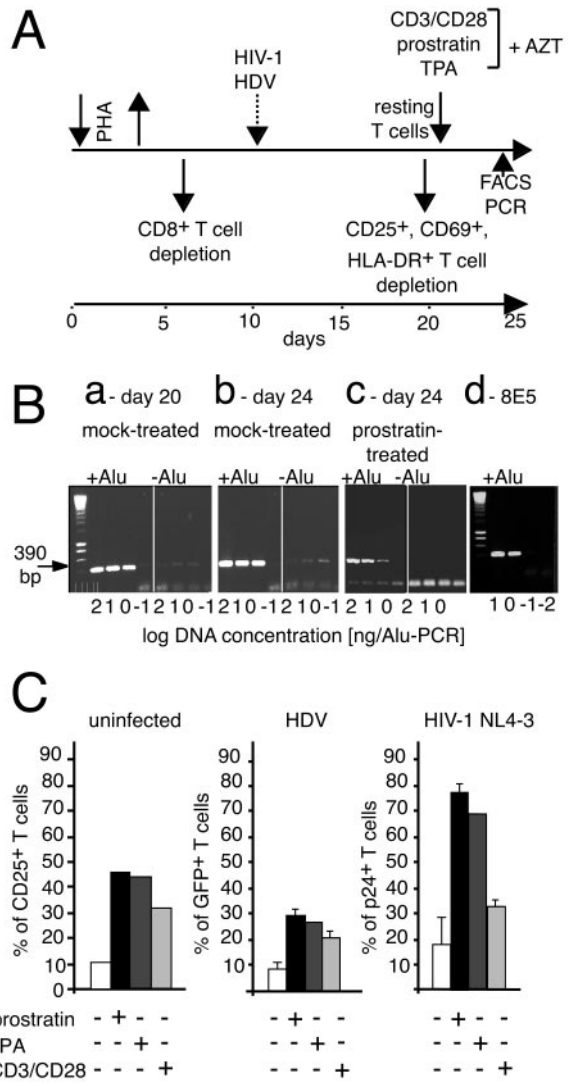


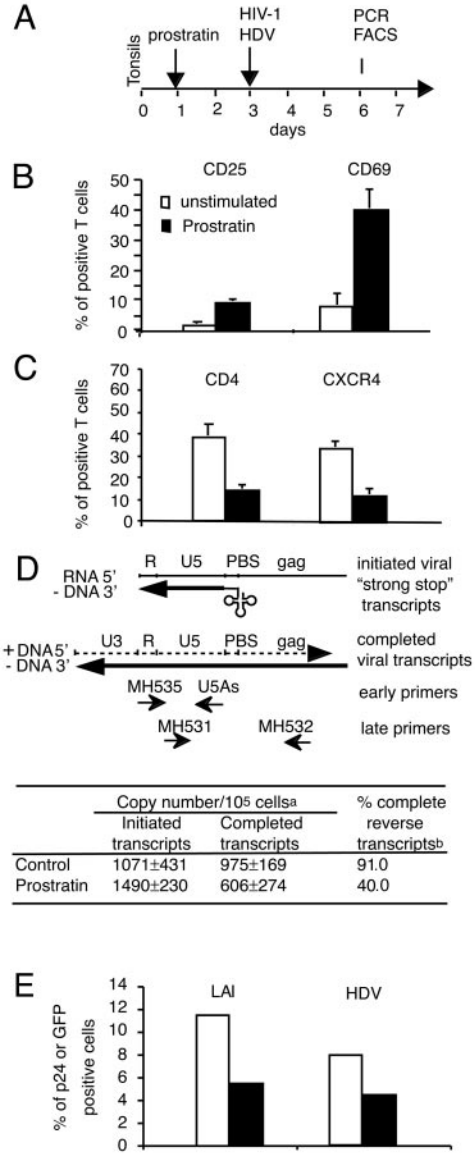
FIG. 3. Effect of prostratin on resting $CD4^+$ T cells infected with HIV-1. PHA-activated PBMC depleted of monocytes, of $CD8^+$ cells, and of $CD25^+/CD69^+/HLA\text{-}DR^+$ cells were inoculated with HIV-1 or HDV on day 10 postactivation. After their return to resting phase (20 days postinfection), residual $CD25^+/CD69^+/HLA\text{-}DR^+$ cells were removed and the remaining cells were treated with 10 μM prostratin, TPA (100 nM), or soluble anti-CD3/CD28 monoclonal antibodies (1 μg/ml) in the presence of 10 μM AZT. Cells were analyzed by means of fluorescence-activated cell sorter (FACS) analysis or Alu PCR on day 3 after application of prostratin. (A) Time schedule of the experiment. (B) Alu PCR of 10-fold serial dilutions of genomic DNA extracted from mock-treated $CD4^+$ T cells (lanes a and b) 20 (lane a) or 24 (lane b) days after PHA stimulation or from prostratin-treated cells at day 24 (lane c). Nested PCR amplifications were conducted with the following primer pairs: a first round with primer pair Alu and L1 (L1 is an antisense primer localized in LTR) (+Alu) and a control round from which the forward Alu primer was omitted (−Alu) were followed by a second round of PCR with HIV-1-specific primers from the U3 part of the HIV-1 LTR. Amplification products were run on agarose gels and visualized by means of ethidium bromide staining. (C) Percentages of noninfected $CD25^+$ cells, HDV-inoculated GFP^+ cells, and HIV-1-infected $p24gag^+$ cells that were left nonreactivated (control) (open bars) or were activated with prostratin (black bars), TPA (dark-gray-shaded bars), or CD3/CD28 (light-gray-shaded bars). The means of at least 4 experiments (14 experiments for prostratin-activated cells) are shown.

integrated provirus by means of Alu PCR of limiting dilutions of genomic DNA extracted from nonstimulated or prostratin-stimulated $CD4^+$ T cells (Fig. 3B). In both nonstimulated and prostratin-stimulated $CD4^+$ T cells, the end point dilution at which Alu PCR signal was detected contained the same quantity of genomic DNA (1-ng aliquots). Therefore, prostratin treatment in the presence of inhibitors of reverse transcriptase did not increase the number of integrated proviruses. In the control genomic DNA extracted from T-cell line 8E5, which harbors one integrated HIV-1 provirus per cellular genome, the end point dilution at which Alu PCR signal was detected also contained 1 ng of DNA (15). This suggests that, on average, one HIV-1 provirus was integrated per genome of resting $CD4^+$ T cells.

Reactivation of resting T cells harboring HIV-1 or HDV provirus with anti-CD3/CD28 antibodies, TPA, and prostratin induced expression of CD25 and increased the proportion of T lymphocytes that expressed the HIV-1 genome (Fig. 3C). In prostratin-stimulated HDV-infected cultures, 31.0% ± 1.5% of these cells were GFP positive versus 9.0% ± 2.5% in unstimulated controls. In HIV-1 NL4-3-infected cultures, prostratin increased the number of $p24gag^+$ cells from 18% ± 10% to 79% ± 4%. Since in these cultures de novo infection was blocked by nevirapine and AZT, the amounts of HIV DNA before and after prostratin stimulation were similar (Fig. 3B). Thus, prostratin releases HIV from postintegration latency, in consistency with the results obtained with PBMC of infected individuals (30) and with mature thymocytes in SCID-hu (Thy/Liv) mice (2, 29). We estimated the minimal percentage of latently infected resting $CD4^+$ T cells as the difference between the percentages of productively infected cells after and before cell activation. We found that at least 22% (31% less 9%) of cells exposed to HDV and at least 61% (79% less 18%) of cells exposed to HIV-1 NL4-3 harbored latent provirus.

Prostratin downregulates CD4 and CXCR4 in lymphoid tissue. We monitored the evolution of expression of cell-surface receptors and activation markers in T cells from blocks of lymphoid tissue pretreated with prostratin and then infected with HIV-1 LAI (Fig. 4A). As with PBMC, prostratin in blocks of lymphoid tissue induced cell surface expression of the activation markers CD25 (9% ± 1% of T cells) and CD69 (40% ± 7% of T cells) (Fig. 4B) and downregulated both CD4 (to levels of 39.5% of the nonstimulated control) and CXCR4 (to levels of 30.6% of the nonstimulated control) (Fig. 4C).

Prostratin blocks completion of reverse transcription of the HIV-1 genome in lymphoid tissue. To quantitate initiated and completed viral reverse transcripts 3 days after infection, we performed real-time PCR (Fig. 4D). Whereas the early primers MH535 and U5As amplified both the initiated viral "strong stop" transcripts and the completed viral transcripts, the late primers MH531 and MH532 amplified only the completed viral transcripts. Therefore, we assessed the copy numbers of initiated transcripts as the difference between copy numbers amplified by early and late primers. Prostratin reduced the completion of the initiated transcripts. The percentages of complete reverse transcripts in prostratin-treated cultures were approximately two times smaller than in control cultures (Fig. 4D). Thus, the diminution of retrotranscription completion also plays a role in prostratin-mediated HIV-1 infection inhibition.



	Copy number/10^5 cells[a]		% complete reverse transcripts[b]
	Initiated transcripts	Completed transcripts	
Control	1071±431	975±169	91.0
Prostratin	1490±230	606±274	40.0

FIG. 4. HIV-1 infection of prostratin-treated lymphoid tissue. Human tonsils (54 to 72 blocks) from each of four donors were stimulated with prostratin and were inoculated with HIV-1 LAI or with HDV 2 days poststimulation. Cells were analyzed by means of flow cytometry and PCR on day 3 postinfection. Infected and control uninfected cultures were gated on $CD3^+$ cells. The percentages of $p24gag^+/CD3^+$ T cells were corrected for background ($p24gag^+/CD3^+$ T-cell counts detected in mock-infected blocks from the same donor). (A) Time schedule of the experiment. (B) Effect of prostratin on the expression of the cell surface activation markers CD25 and CD69 on tissue T cells. (C) Effects of prostratin on the expression of the HIV-1 receptors CD4 and CXCR4 on tissue T cells. (D) Effect of prostratin on completion of reverse transcription in tonsillar tissue infected with HIV-1 LAI 3 days after stimulation, as determined from real-time PCR 3 days postinfection. Second column from the left: viral copy numbers and cell equivalents were determined by comparison with the known quantity of pNL4.3 and with actin standards, respectively. Third column: the copy numbers of initiated transcripts were assessed as the difference of copy numbers amplified by early and late primers. Note that early primers amplify both initiated and completed viral transcripts. Fourth column: the percentage of complete reverse transcripts is calculated as the ratio of completed to initiated transcripts × 100. R, short repeat. (E) Effect of prostratin on the expression of HIV-1 and HDV in tissue T cells as determined with p24gag and GFP, respectively.

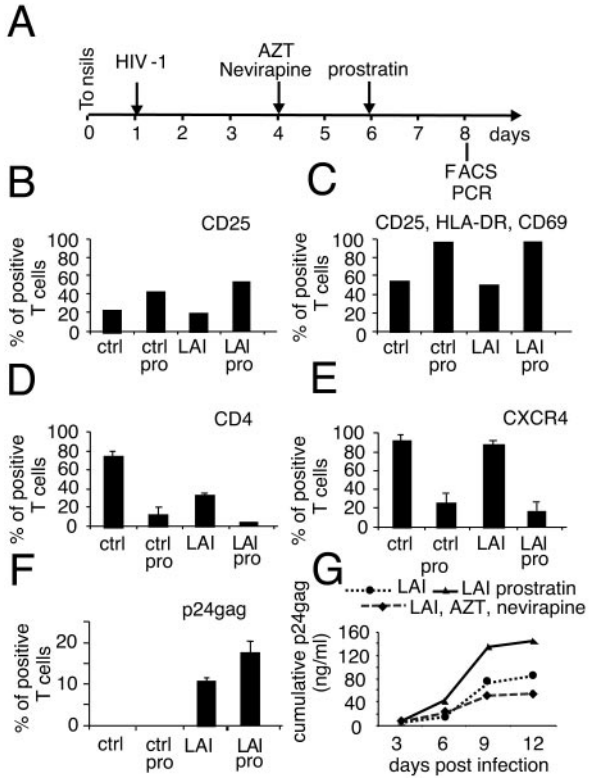


FIG. 5. Effect of prostratin on lymphoid tissue infected with HIV-1. Human tonsils (54 to 72 blocks) from each of five donors infected ex vivo with HIV-1 NL4-3 were treated with 10 μM AZT and 5 μM nevirapine on day 3 postinfection and stimulated with 10 μM prostratin (pro) on day 5 postinfection. Cells were analyzed by means of flow cytometry and PCR on day 7 postinfection. Ctrl, control. (A) Time schedule of the experiment. FACS, fluorescence-activated cell sorter. (B to E) Percentages of T cells in HIV-1-infected and prostratin-treated human lymphoid tissue expressing the following markers: CD25 (B); CD25, CD69, and HLA-DR (C); CD4 (D); CXCR4 (E); p24gag (F); and p24gag in culture medium (G). Infected and control uninfected cultures were gated on $CD3^+$ cells. The percentages of $p24^+/CD3^+$ T cells were corrected for background ($p24^+/CD3^+$ T-cell counts detected in mock-infected blocks from the same donor).

In consistency with the blockade of the completion of reverse transcription of its genome, HIV-1 was expressed in a significantly smaller proportion of prostratin-activated T cells than in untreated controls (5.7% ± 0.4% versus 12.0% ± 0.8%; $n = 4$, $P = 0.003$) (Fig. 4E). Interestingly, prostratin reduced the expression of HDV pseudotyped by VSV protein G in T cells to the same extent as it did the expression of HIV-1 (Fig. 4E), while HDV enters T cells by a CD4- and CXCR4-independent mechanism.

Prostratin activates viral replication in HIV-infected lymphoid tissue. Blocks of human tonsils (54 to 72 blocks) from each of five donors were infected ex vivo with HIV-1 LAI and treated with 10 μM AZT and 5 μM nevirapine on day 3 postinfection. Tissue blocks were stimulated by the addition of 10 μM prostratin on day 5 postinfection (Fig. 5A). As with PBMC, prostratin stimulated the expression of the activation markers CD25 and CD69 on tissue cells (Fig. 5B, 5C). Expression of HIV receptors CD4 and CXCR4 was reduced in prostratin-treated tissues (Fig. 5D and E). However, in this case the

proportion of T lymphocytes that expressed HIV in the presence of AZT and nevirapine was increased in prostratin-activated tissue in comparison with the results seen with matched tissues not treated with prostratin (Fig. 5F). The prostratin-induced increase of HIV replication was also detected from the increased accumulation of p24gag in culture medium bathing the tissue blocks (Fig. 5G).

DISCUSSION

Restriction of both HIV-1 replication and reactivation of HIV-1 latency in lymphoid tissue, the primary site of HIV-1 infection in vivo, is of paramount importance to the development of new therapies. The effect of prostratin on reactivation of HIV-1 latency in lymphoid tissue is particularly important, because the latent reservoir is formed predominantly in this compartment of the lymphatic system. Whereas previous studies investigated either inhibition of HIV replication or reactivation of HIV provirus from its latency, we studied both seemingly incompatible processes inherent in the activity of prostratin in two systems, PBMC and lymphoid tissues.

Our results show that prostratin significantly restricts primary resting $CD4^+$ T-cell susceptibility to HIV-1 infection in PBMC and in lymphoid tissue. Here, we demonstrate that prostratin suppresses HIV-1 infection along several pathways: (i) it downregulates HIV-1 receptor CD4 and coreceptor CXCR4; (ii) it inhibits viral entry into the cells, probably as a result of this receptor-coreceptor downregulation; and (iii) it inhibits the completion of reverse transcription. Whereas downregulation of CD4 molecules by prostratin has been observed in PBMC and in several T-cell lines (24, 25, 30), we detected this phenomenon in $CD4^+$ T lymphocytes in the lymphoid tissue. Diminished expression of CXCR4 in prostratin-treated cells has been predicted on the basis of study of the transcriptome of the THP-1 cell line but has not been demonstrated at the protein level (24, 25, 30).

Whereas pretreatment with prostratin dramatically inhibited virus entry into $CD4^+$ T cells from PBMC (Fig. 1I), it did not reduce levels of initiated reverse transcripts in lymphoid tissue 3 days postinfection (Fig. 4D). Similar levels of initiated reverse transcripts in control and in prostratin-treated tissues could be caused by more-rapid depletion of infected $CD4^+$ T cells in the control tissue. While prostratin is insufficient to activate quiescent $CD4^+$ T cells from PBMC to overcome an intrinsic block in reverse transcription (29), our present data directly show that prostratin inhibits completion of reverse transcription. Quantification of incomplete reverse transcription in $CD4^+$ T lymphocytes infected first with HIV and then stimulated with prostratin is difficult to interpret, because the results are biased by the rapid formation of integrated proviruses in stimulated cells. Thus, prostratin seems to interfere with both entry and postentry events in the HIV-1 infection cycle. This interference was confirmed in our experiments with HDV pseudotyped with VSV protein G, which requires neither CD4 nor CXCR4 for cell entry. Nevertheless, prostratin inhibited HDV transduction at a postentry step not yet precisely defined.

Prostratin has previously been reported to inhibit the replication of HIV-1 in a variety of acutely but not persistently infected cell systems (24, 25, 30). Our results demonstrate that prostratin reactivates HIV-1 from pre- and postintegration latency in PBMC and in lymphoid tissue. This is evident from the increase in p24gag in the medium as well as from an increase in the number of $p24gag^+$ T cells. This result could alternatively be explained by proliferation of HIV-infected cells in prostratin-treated tissues. However, that is not the case, since unlike the results seen with activation by PHA or anti-CD3/CD28 antibodies, no cell division occurred after prostratin stimulation. Thus, prostratin does not have a mitogenic effect and the increase of viral production in HIV-1-infected prostratin-treated cultures is the result of the reactivation of expression of latent virus. This conclusion is further supported by the results obtained with HDV, which establishes latency but is unable to form infectious progeny. Prostratin rescues HDV from its latent state.

HIV-1-infected resting $CD4^+$ T cells generated in vitro were used as a model of postintegration latency. Up to 30% of these cells are infected productively (20, 34). Several soluble factors responsible for productive infection of resting $CD4^+$ T cells have been identified recently (41, 42, 46). Among them, IL-2, a cytokine necessary for the survival of cells in cultures used in our experiments (20), is apparently an essential factor for productive infection of resting $CD4^+$ T cells with HIV-1 (46). These cells are a convenient model for in vitro studies but also may have relevance in vivo: whereas resting $CD4^+$ T cells from peripheral blood of aviremic long-term HIV-infected individuals treated with HAART harbor predominantly latent provirus (9, 27), a high proportion of resting $CD4^+$ T cells in PBMC of viremic patients (9) and in lymphoid tissue of HIV-1-infected individuals and simian immunodeficiency virus-infected monkeys (40, 52) are infected productively.

The percentages of latently infected resting $CD4^+$ T cells in the postintegration latency model, as assessed on the basis of reactivation of latent provirus, exceeded the percentage of productively infected cells in the same culture by two to three times. The real proportion of latently infected cells could actually be higher because of the unknown efficiency of reactivation of HIV latency. According to a minimum estimate, prostratin reactivated HIV-1 replication in 22 to 66% of resting latently infected $CD4^+$ T cells. Whereas HIV-1 replication was reactivated in a comparable proportion (25 to 38%) of latently infected thymocytes generated in SCID-hu (Thy/Liv) mice (2), only 1% of the HIV-1 DNA-positive resting $CD4^+$ T lymphocytes of aviremic individuals could be induced to upregulate HIV-1 mRNA after cellular activation (27). This indicates that most of the proviral DNA in resting $CD4^+$ T cells in peripheral blood of infected patients either carries intrinsic defects precluding expression of the HIV genome or is subjected to an epigenetic control mechanism. Pion et al. (35, 36) and Sanchez et al. (37) have previously demonstrated a high proportion of truncated proviruses in PBMC and lymph nodes of HIV-1-infected individuals and simian immunodeficiency virus-infected macaques but not in in vitro-infected $CD4^+$ T cells. Thus, the absence of selective mechanisms leading to the accumulation of defective and expression-blocked proviruses in vivo can cause the great majority of proviruses in in vitro HIV-1-infected $CD4^+$ T cells to be reactivated by prostratin or PHA.

In the postintegration latency model, PHA activation precedes generation of HIV-1-infected resting $CD4^+$ T cells in

vitro and subsequent reactivation of HIV-1 from its latency (Fig. 3). Higher induction of HIV-1 gene expression and of CD25 expression by stimulation with prostratin than by costimulation with anti-CD3/CD28 antibodies suggests that PHA preactivation can cause partial anergy of the CD3 receptor. Indeed, anti-CD3/CD28 costimulation of cells not preactivated by PHA resulted in a higher efficiency of CD25 induction than did stimulation with prostratin (reference 29 and our unpublished results). The potential for anergy of the CD3 receptor could play an important role in the failure to purge the latent HIV-1 reservoir in vivo with anti-CD3 antibodies or by contact with a natural antigen of latently infected CD4 T cells (12, 47). It highlights an urgent need to search for alternative HIV-1-reactivating agents.

Reactivation of HIV-1 from postintegration latency has recently been observed in PBMC of HAART-treated patients (30) and in mature thymocytes in SCID-hu (Thy/Liv) mice (29). However, the precise mechanism of transduction of the prostratin-induced cell surface signal into the nucleus and of the resulting reactivation of latent provirus remains only partially understood (45). Data from other laboratories show that prostratin does not induce cell cycling (29). Bovine FCS used in our experiments to supplement tissue culture media could be responsible for a low but distinct expression level of CD25 in human $CD4^+$ T cells negatively selected for the CD25 activation marker. These preactivated cells may be making a significant contribution to the effects of prostratin. We have found that the presence of prostratin inhibits the division of PHA-activated $CD4^+$ T cells but strongly upregulates the activation markers CD69 and CD25, the α chain of the IL-2 receptor. CD69 is expressed on a higher percentage of prostratin-stimulated T cells than is CD25; this differential is consistent with the classing of CD69 as an "early" activation marker, whereas because of the sustained expression of CD25 it (like HLA-DR) is classed as a "late" activation marker (11, 26). Whereas induction of CD25 seems to be directly related to the activation of protein kinase C signaling, the cytostatic effect of prostratin on PHA-stimulated cells remains to be explained.

Prostratin releases HIV from preintegration latency as well as from postintegration latency. While HIV-1 uptake occurred in untreated resting $CD4^+$ T cells, the viral integration was inefficient and the number of $p24gag^+$ cells was low when HIV-1 infection was not followed by subsequent stimulation (Fig. 1I). In cultures treated with prostratin 1 day after virus inoculation, the fraction of infected cells increased 20-fold in spite of the fact that de novo infection with HIV-1 was blocked by AZT and nevirapine and also in spite of the use of a replication-incompetent virus.

These results suggest on the one hand that prostratin promotes the integration of completed copies of the HIV-1 genome into host DNA and their further expression and on the other hand that it blocks early steps of the HIV-1 viral cycle (HIV-1 uptake and completion of reverse transcription of the HIV-1 genome). To study reactivation of HIV-1 latency, we used a system of HIV-infected human lymphoid tissue ex vivo. Whereas virtually all target cells of HIV-1 infection are present in tonsillar tissue, only $CD4^+$ T lymphocytes are infected productively with the X4 HIV-1 variants NL4-3 and LAI used in our experiments. Because prostratin may not thoroughly penetrate the blocks of lymphoid tissue ex vivo, its effects as determined in our experiments can lead to underestimation of its actual activity. We have shown that in this system prostratin both blocks HIV entry and seems to release viral latency, since it activates replication in spite of the blockade of de novo infection.

In conclusion, our work, together with earlier published data (2, 24, 29, 30), suggests that prostratin is a potent inducer of latent HIV from resting T cells and an inhibitor of HIV infection de novo.

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