Host factor pleiotrophin induces human immunodeficiency virus type 1 replication associated with inflammatory cytokine expression

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Pleiotrophin (PTN) is a polypeptide that belongs to a family of heparin-binding growth factors; it displays mitogenic activity for a wide variety of cells. In a previous study, we reported that PTN induces the stimulation of expression of inflammatory cytokines, including tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6, in quiescent human peripheral blood mononuclear cells (PBMCs) through B-lymphocyte binding. These results emphasize the importance of PTN in the regulation of inflammatory processes. Moreover, using in vitro infection of PBMCs or using PBMCs from AIDS patients, we showed that PTN was sufficient to induce human immunodeficiency virus type 1 (HIV-1) replication. Moreover, neutralization of TNF-α, IL-1β and IL-6 suppressed HIV replication in PTN-stimulated PBMCs. As these cytokines are potent upregulators of virus expression, these results should prove useful in investigating the role of PTN as a host factor in the regulation of pathological disorders in HIV-1 infection. Identification of this host factor could be important for understanding HIV disease and designing therapeutic approaches.

INTRODUCTION

Productive infection of primary T cells requires the assistance of several factors for provirus establishment and virus replication. Activation of unknown host factors that potentiate human immunodeficiency virus type 1 (HIV-1) replication and dissemination has not been delineated fully. The ability of HIV-1 to establish persistent infection is essentially dependent on the cellular signals that regulate virus replication within target cells. Replication of HIV-1 is regulated by virus-encoded regulatory proteins, as well as by a variety of cellular factors including cytokines. This viral infection reflects a complex balance of viral and host factors. It has been reported that pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6, are potent upregulators of virus production in mononuclear cells (Fauci, 1993). Moreover, autocrine/paracrine regulation of HIV replication by endogenous cytokines occurs in acutely or chronically infected cell lines, as well as in primary cells derived from patients (Butera et al., 1993; Kinter et al., 1995; Mace et al., 1989; Poli et al., 1990; Tadmori et al., 1991).

Pleiotrophin (PTN) and its structurally related polypeptide, MK, belong to the family of heparin-releasable polypeptides. This family of proteins includes lipoprotein–lipase tissue-factor pathway inhibitor, extracellular superoxide dismutase, histaminase platelet factor 4 and TNF-binding protein 1, and contributes to vascular homeostasis (Novotny et al., 1993). Our initial finding showing that PTN is an angiogenic growth factor (Laaroubi et al., 1995) that is synthesized and located in activated endothelial cells prompted us to investigate a possible function of PTN for blood cells. In a previous report, we investigated this possibility and evaluated the in vitro activity of this growth factor on freshly isolated peripheral blood mononuclear cells (PBMCs) (Achour et al., 2001). We showed that PTN induces the production of inflammatory cytokines, including TNF-α, IL-1β and IL-6, using PBMCs as target cells (Achour et al., 2008).

As the replication of HIV-1 is regulated by a variety of cellular factors, including pro-inflammatory cytokines, we investigated the effects of PTN on HIV-1 replication in human PBMCs. Elucidation of the mechanisms by which a
host factor such as PTN regulates HIV-1 infection within target cells will advance our understanding of host–virus interactions significantly.

RESULTS

Primary and laboratory isolates of HIV replicate in PTN-stimulated PBMCs

As pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are potent upregulators of HIV-1 expression, we postulated that PTN might be associated with virus replication. The efficiency of replication of primary and strain isolates was evaluated using PBMCs from healthy individual donors that were stimulated with either phytohaemagglutinin A (PHA) or PTN. The activated cells were then infected, washed and expanded in growth medium containing recombinant IL-2. As shown in Fig. 1a(i), primary isolates replicated with comparable efficiency in both PHA blasts and PTN-stimulated PBMCs. However, increased efficiency of virus replication in PHA blasts [Fig. 1b(i)], compared with PTN-stimulated PBMCs from the same individuals, was observed with the T-lymphotropic HIV-1 IIIB strain. This increase of HIV-1 replication in PHA blasts compared with PTN-stimulated PBMCs might be caused by a preferential tropism for T lymphocytes associated with cell stimulation with a potent mitogen such as PHA. It is noteworthy that, after 10 days culture, cell proliferation in PHA-activated cells was significantly higher than that in PTN-stimulated cells (60–100 % more; \( P<0.01 \)) [Fig. 1a(ii), b(ii)]. Analyses by flow cytometry demonstrated that both CD4 and CD8 T lymphocytes were expanded equally by PTN (data not shown). In that way, the cell proliferation in total PBMCs could be relevant for virus replication. Comparatively, no cell proliferation was observed when PBMCs were cultured using growth medium alone.

Neutralization of TNF-α, IL-1β and IL-6 suppressed HIV replication in PTN-stimulated PBMCs

Stimulation with certain cytokines has previously been demonstrated to upregulate HIV replication in different cell cultures. In addition, neutralization of TNF-α, IL-1β or IL-6 resulted in not complete, but significant suppression

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Fig. 1. Effect of PTN on HIV-1 expression in PBMCs originating from HIV-1-seronegative individuals. The efficiency of infection with a primary HIV-1 isolate [a(i)] or strain HIV-1 IIIB isolate [b(i)] was determined in the absence (▲) or the presence of PTN (1 \( \mu \text{g ml}^{-1} \)) (●) or PHA (4 \( \mu \text{g ml}^{-1} \)) ( ■) supplemented to PTN-activated cells (○) or PHA-activated cells (□) with a mixture of anti-cytokine Abs, including anti-IL-1β, anti-IL-6 and anti-TNF-α. Culture supernatants were harvested at days 3, 7 and 10 and tested for RT activity as described in Methods. The number of viable cells as assessed by trypan blue exclusion is represented in [a(ii)] for infection with the primary isolate and in [b(ii)] for infection with the strain isolate. Results are representative of four separate experiments. Each point represents the mean of three experimental values; error bars indicate SEM.
of HIV replication (Poli & Fauci, 1993). Therefore, these pooled anti-cytokine antibodies (Abs) were tested for their capacity to interfere with HIV replication in PTN-stimulated cultures. PBMCs were incubated with anti-cytokine Abs at the time of infection and cultures were supplemented with fresh medium containing the Abs at each passage. PBMCs from at least five different donors were used in independent experiments for the pooled anti-cytokine Abs tested. Neutralization of TNF-α, IL-1β and IL-6 together resulted in significant suppression of HIV expression (Fig. 1) compared with PTN-stimulated cells incubated without anti-cytokine neutralizing Abs (84 and 87 % of virus inhibition at respectively 7 and 10 days culture; P<0.001). Comparatively, when stimulated cells were incubated with control goat serum, no virus inhibition occurred (data not shown). This effect was not a consequence of suppression of cellular proliferation, as determined by blue dye exclusion criteria [Fig. 1a(ii), b(ii)]. As shown in Fig. 1, no clear evidence of suppression of HIV replication was observed in parallel cultures of PHA blasts treated with the anti-cytokine Abs, as described by others in a previous study (Poli et al., 1990).

Identification of the cells involved in PTN-dependent HIV-1 replication

To determine the phenotype of cells involved in PTN-dependent virus production, we initially separated PBMCs into a population of cells that adhered to plastic, i.e. mainly the monocytes, and those that were non-adherent, i.e. both B lymphocytes (CD19+ ) and T lymphocytes (CD3+ ). After cell activation with PTN, the cells were infected with 100 TCID₅₀ (50 % tissue culture infectious dose) of a clinical HIV-1 isolate corresponding to a non-syncytium-inducing (NSI) phenotype. None of these cell populations showed any substantial virus production, even after 6 days culture (Table 1), in contrast to total PBMCs. When PHA is used as a positive control of cell activation, high virus production was observed in total PBMCs. This production was lower when non-adherent cells were depleted. In total PBMCs, PTN induced HIV-1 production at drug concentrations ranging from 10 to 1000 ng ml⁻¹ (peak, 100 ng ml⁻¹) (Table 1). To determine whether the adherence period can induce changes in PTN stimulation, we cultured PBMCs in the absence of PTN or PHA for the same period of time as the adherence protocol. The results in Table 1 indicated that the PTN-mediated effect on virus replication was not affected. When non-adherent cells and adherent cells were mixed after PTN stimulation and finally infected, we still observed a PTN-mediated effect on virus replication.

We next determined whether inflammatory cytokine production was defective in each fraction of cells (adherent versus non-adherent) after PTN stimulation. Therefore, we identified by fluorescence-activated cell sorting (FACS) analysis the cells involved in PTN-mediated cytokine expression. The results illustrated in Fig. 2 show that adherent cells (CD14+) showed no substantial cytokine production after 3 days culture, in contrast to total PBMCs (35 % of cells expressed TNF-α, 12 % IL-1β and 62 % IL-6). In non-adherent cells, only production of TNF-α (18 %) and IL-6 (22%) was observed. Moreover, when adherent cells were mixed with the non-adherent cell fraction after PTN stimulation, substantial production of TNF-α (32 %), IL-1β (10 %) and IL-6 (52 %) was observed.

Effect of PTN on PBMCs from naturally infected patients

The role of PTN was also investigated using naturally HIV-1-infected cells. The ability of IL-2-stimulated PBMCs to support HIV replication has been documented previously (Kinter et al., 1995). As AIDS is characterized by a chronic

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Amount of HIV-1 induced by:</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>10 ng ml⁻¹</td>
</tr>
<tr>
<td>Total PBMCs (CD3⁺, CD19⁺, CD14⁺)</td>
<td>210 ± 70</td>
</tr>
<tr>
<td>Adherent cells (CD14⁺) (plate adherence for 12 h)</td>
<td>222 ± 75</td>
</tr>
<tr>
<td>Non-adherent cells (CD3⁺, CD19⁺, CD14⁺)</td>
<td>284 ± 65</td>
</tr>
<tr>
<td>Adherent + non-adherent cells (after PTN stimulation)</td>
<td>251 ± 56</td>
</tr>
<tr>
<td>Total PBMCs (12 h and next PTN stimulation)</td>
<td>235 ± 90</td>
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</tbody>
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* P<0.001 vs control.
** 5 × 10⁵ total or separated cells were activated with PHA or recombinant PTN (10, 100 or 1000 ng ml⁻¹) and thereafter infected.
†Adherent and non-adherent cells were mixed after PTN stimulation.
§PBMCs were cultured in the absence of stimulation for 12 h (time of adherence protocol) and then PTN/PHA-stimulated before virus infection.

Table 1. Induction of HIV-1 in different cell types

Virus expression was measured by RT assay on day 6 of culture as described in Methods. Each value represents the mean ± SEM of data obtained in cells obtained from three donors with three different samples.
immune activation, we postulated that PTN alone, without the association of recombinant cytokines, could be efficient for virus production in cells derived from an HIV-1 patient. As illustrated in Fig. 3a(i), when cells were stimulated with PTN alone, virus production was significantly higher than in the control cells, which were not stimulated, after only 3 days culture (100% higher at 10 ng ml\(^{-1}\), 500% at 100 ng ml\(^{-1}\) and 400% at 1 \(\mu\)g ml\(^{-1}\)) and respectively 285, 800 and 622% at 6 days [Fig. 3b(i)]. Moreover, neutralization of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 together resulted in significant inhibition of HIV expression \((P<0.005)\) [Fig. 3a(i), b(i)]. The observed effects were not a consequence of cell mortality or inhibition of cell multiplication at either 3 days [Fig. 3a(ii)] or 6 days [Fig. 3b(ii)] culture. Compared with the control, PTN induced low but significant augmentation of cell number \((P<0.005)\). This increase of cell proliferation was 140% at 10 ng ml\(^{-1}\), 75% at 100 ng ml\(^{-1}\) and 8% at 1 \(\mu\)g ml\(^{-1}\) after 6 days culture. To specify the role of PTN in the absence of recombinant IL-2, PBMCs derived from six additional patients were studied. The level of virus expression increased specifically after 3 days culture. As shown in Table 2, consistent changes in virus production in the treated PBMCs, as evaluated by serial measurement of p24 antigen, were detected during the culture. At 3 days culture, cells treated with PTN exhibited an increase in p24 antigen levels. However, at day 1, p24 antigen levels were comparable to untreated cells (Table 2). An enhancement of HIV expression was observed in cultures of PTN plus IL-2-stimulated lymphocytes, and cells cultured with IL-2 alone did not exhibit an amplification of virus expression. No differences in the PTN, IL-2 and PTN + IL-2 stimulation of HIV production were observed between the patients with low viral load (P5, P10, P11, P12) and those with higher viral loads (P16, P17).

**DISCUSSION**

In the present study, we described a culture system of PTN-stimulated PBMCs, in which HIV-1 infection could be established using either primary or laboratory virus isolates. Primary isolates of HIV-1 replicated with comparable efficiency in PTN-stimulated cultures and cultures stimulated with a non-physiological stimulus, such as PHA. However, the laboratory strain of HIV-1 (IIIB), a T-cell-tropic strain, replicated with greater efficiency in PHA blasts. This may be explained by the fact that laboratory strain IIIB replicated more efficiently in T lymphocytes. Although it is generally admitted that primary strains do not exhibit strict tropism for monocytes or lymphocytes, they present differences in their ability to infect these two cell types in vitro (Zack et al., 1990). Moreover, we have also shown that PTN alone can induce virus expression, which was amplified in the presence of recombinant IL-2, in PBMCs originating from AIDS patients. We used fresh cells without monocyte enrichment, and no HIV infection
occurred in the presence of IL-2 alone. Thus, exogenous IL-2 can amplify PTN stimulation and HIV-1 expression was observed in the presence of this factor.

Autocrine/paracrine regulation of virus expression by proinflammatory cytokines is established either in infected cell lines or in primary patients’ PBMCs (Kinter et al., 1995). In a previous study, we reported that PTN induces proliferation in PBMCs, with a maximal effect at 1 μg ml⁻¹ and little activity at 100 ng ml⁻¹ (Achour et al., 2001). In the current manuscript, the effects seen on HIV replication were at 10–1000 ng ml⁻¹ in the presence of the T-cell growth factor IL-2. This result suggested that PTN would need to provide a growth stimulus for lymphocytes in addition to inflammatory cytokines for long-term culture. Thus, this system could provide a reliable approach for

Table 2. Virus expression in PBMCs derived from HIV-1-infected individuals following treatment with PTN

<table>
<thead>
<tr>
<th>Patient</th>
<th>1 day culture</th>
<th>3 days culture</th>
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<tbody>
<tr>
<td></td>
<td>(−)</td>
<td>IL-2</td>
</tr>
<tr>
<td>P5</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>P10</td>
<td>25</td>
<td>28</td>
</tr>
<tr>
<td>P11</td>
<td>29</td>
<td>33</td>
</tr>
<tr>
<td>P12</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>P16</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td>P17</td>
<td>38</td>
<td>41</td>
</tr>
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*P<0.005.
endothelial cells to potentiate the immune response during the inflammatory process.

When separated (adherent or non-adherent) cells were used, no virus production was observed. Moreover, PTN stimulated HIV replication when non-adherent (B cells and T cells) and adherent (monocytes) cells were mixed after PTN stimulation. Virus production was dependent on the presence of T cells, B cells and monocytes, supporting the hypothesis of cooperation between the three cell populations. PTN-mediated cytokine production (TNF-α, IL-6 and IL-1β) was also dependent on the presence of adherent and non-adherent cells. Comparatively to virus infection experiments, in the PTN-binding assay, we showed that only B cells could bind the PTN molecule (Achour et al., 2008). Interaction between B and T cells is an important element of the immunological response. It allows T cells and their counterparts, B cells, to assume different functions. It initiates multiple signals that provide a model of intracellular signalling. Taking our results into account, we postulate that the PTN molecule, by binding to B cells, might facilitate the mechanism leading to signal activation and cytokine production necessary for virus production. Further studies are necessary to identify the PTN receptor on B cells in relation to the signal activation necessary for cell activation and cytokine production. It is reported that PTN induces endothelial cell proliferation and binds to vascular endothelial growth factor (Polykratis et al., 2005). Moreover, HIV infection of tissues is probably mediated by transmission of HIV-infected cells across the endothelial cell barrier. Action of PTN in the vascular microenvironment may contribute to the dissemination of HIV into tissue and, therefore, may lead to enhanced disease pathology. Additional studies would be required to determine PTN expression in endothelial cells and in lymph nodes derived from AIDS patients.

Despite numerous studies, many fundamental issues in HIV-1 pathogenesis remain poorly understood. It has long been considered that productive HIV-1 infection and cell proliferation are dependent events in primary T cells (Zack et al., 1990). Productive HIV-1 infection of primary T cells is blocked in cells, depending upon their activation status. Releasing this step to HIV-1 replication requires states of T-cell activation that include induction of specific host cellular factors (Spina et al., 1995; Zack et al., 1990). Although T-cell activation and induction of host cellular factors are critical for HIV-1 provirus establishment and replication in primary human T cells, these cellular factors have not been identified fully (Kinoshita et al., 1998). Studies performed using chronically infected cell models demonstrate that pro-inflammatory cytokines stimulate HIV-1 expression from latency. Mechanistically, these cytokines activate HIV-1 expression from latency via receptor-mediated second messenger signal transduction, resulting in NF-κB activation (Beg et al., 1993). TNF-α and IL-1β have both been shown to activate the cellular transcription factor NF-κB, a potent transactivator of the HIV long terminal repeat. Our observations with anti-cytokine Abs suggest that the cytokines TNF-α, IL-1β and IL-6 are consistently involved in enhancing HIV-1 replication in PTN-stimulated PBMCs. This study is the first to demonstrate such a phenomenon induced by a heparin-releasable polypeptide. Furthermore, the identification of PTN as a host factor required for Tat activity may identify potential new targets for therapeutic intervention against HIV-1 latency.

It was reported that PTN inhibits HIV attachment to the cell surface through an interaction with surface-expressed nucleolin (Said et al., 2002). In addition, PTN cell internalization occurs through interaction with cell-surface nucleolin (Said et al., 2002). In this study, only the role of PTN on cell attachment of HIV is explored. Taken together, our results indicate that the role of PTN in HIV replication is not a direct effect of PTN binding, but rather a consequence of PTN’s induction of inflammatory cytokines. The role of cell-surface nucleolin, as well as other PTN receptor(s), in inflammatory cytokine expression is currently under investigation.

In conclusion, the results described in this study show, to our knowledge for the first time, that the host angiogenic factor PTN could induce HIV-1 replication in infected mononuclear cells. Our results could advance understanding of how PTN may actively participate in the activation and dissemination of virus.

METHODS

Production and purification of human recombinant PTN. Escherichia coli BL21 pLys cells (Novagen) were transformed with the human PTNpETHH8 plasmid (kindly provided by P. Bohlen, ImClone Systems Inc.) (Achour et al., 2008). Purified human recombinant PTN was found to be active by a neurite outgrowth assay, performed as described previously (Achour et al., 2008). Contaminants of pyrogen were removed from the recombinant PTN solution by immobilized polymyxin B (Affinity Pack Detoxi-Gel; Pierce, Perbio Sciences). For the detection of endotoxin, we used the Limulus amoebocyte lysate (LAL) assay. In our preparation, the endotoxin level was <1.0 endotoxin units (µg PTN)-1, as determined by the LAL method. Furthermore, to demonstrate the purity of PTN, we used in our assays an inhibitor of endotoxins, such as polymyxin B (Sigma) (Achour et al., 2008).

Cells and culture conditions. Heparinized venous peripheral blood was obtained from volunteer healthy individuals or from HIV-1-infected individuals (Achour et al., 2003). The study was performed in accordance with local ethical committee standards as reported previously (Achour et al., 2003, 2008). PBMCs were isolated by Ficoll-Hypaque density-gradient centrifugation and were cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics (100 U penicillin ml-1 and 100 µg streptomycin ml-1), all provided by Sigma Aldrich). Thereafter, the cells were resuspended and cultured in complete medium. Cell seeding consisted of 1 × 10⁶ viable cells ml-1 for culture. Cells were grown at 37°C in vented upright 3065 flasks (Costar) containing 5 ml complete medium. To obtain non-adherent and adherent cells, PBMCs were subjected to plate adherence for 12 h. Adherent cells contained >93% CD14+ monocytes and were determined on their expression of CD86+, CD32+, CD14+, CD11c+, CD83+, CD4+ and

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Flow-cytometry analysis. CD4+ and CD8+ T-cell counts, as well as counts of CD19+ and CD14+ cells, were assessed by flow-cytometry analysis (FACSscan; BD Biosciences). Monoclonal antibodies (mAbs) to the following T-cell surface markers were used: CD4–FITC (fluorescein isothiocyanate), CD8–PE (phycoerythrin), CD4–PerCP (peridinin–chlorophyll protein complex), CD8–PerCP, CD19+ and CD14+ (Becton Dickinson), and CD86–FITC, CD32–FITC, CD11c–FITC and CD83–PE (BD PharMingen). In each analysis, 10,000 events were analysed. Each experiment was repeated at least twice and the results provided are from five representative experiments.

Intracellular cytokine staining. Cells were fixed in a final concentration of 1% formaldehyde at 4°C overnight. Subsequently, they were washed in PBS and then in PBS containing 2% FCS and 0.1% saponin (Sigma). PE-conjugated anti-IL-6, anti-TNF-α or anti-IL-1β mAb (Becton Dickinson Immunocytometry Systems) in 30 µl PBS/saponin was used to stain each sample for 60 min (30 min at 4°C followed by 30 min at room temperature). A negative control was prepared by incubating cells with an isotype-matched control Ab.

In vitro infection of PBMCs derived from healthy individuals. Pellets corresponding to 5 × 10^6 PBMCs were infected either with 100 TCID50 of clinical HIV-1 isolate corresponding to an NS1 phenotype or with purified IIIB T-lymphotope-tropic (X4) HIV-1 LAI isolate. After 1 h incubation at 37°C, PBMCs were then washed and activated or not either by 1 µg PTN ml⁻¹ or by 4 µg PHA ml⁻¹ (Sigma Aldrich) for 1 h. The same methods of virus production were observed when PBMCs were first stimulated with PTN and then infected. Thereafter, PBMCs were cultured in growth medium complete medium supplemented with 20 U recombinant IL-2 ml⁻¹ (Boehringer Ingelheim), aiming to expend PHA- and PTN-activated cells in the presence or absence of pooled anti-human cytokine neutralizing Abs (5 µg ml⁻¹ of each Ab). The following Abs were obtained from R&D Systems: goat anti-IL-1β (AF-201-NA), goat anti-TNF-α (AF-210-NA) and goat anti-IL-6 (AF-206-NA). As a control, serum from a non-immunized goat was used. Cell seeding consisted of 1 × 10^4 viable cells ml⁻¹ for 3 days of culture. At each passage, the cells were washed to collect supernatants and cultured in growth medium. Cells were incubated at 37°C in vented upright Costar 3065 flasks containing 5 ml complete medium.

PTN stimulation of PBMCs derived from AIDS patients. Cells from an AIDS patient (P5) were activated with different concentrations of PTN ranging from 10 to 10^5 ng ml⁻¹ and cultured in the presence or absence of pooled anti-human cytokine neutralizing Abs, including anti-IL-1β, anti-TNF-α and anti-IL-6. At the time of the study, the donor was asymptomatic and had been infected for approximately 5 years. His CD4 count was low (210 cells mm⁻³) and his viral load had remained relatively low [5000 RNA copies (ml plasma)]⁻¹. In a set of experiments, PBMCs originating from different, chronically HIV-1-infected individuals were conditioned with medium as control, with 20 U IL-2 ml⁻¹ (Boehringer GMBH) or stimulated with PTN (100 ng ml⁻¹) in the presence or absence of IL-2 and subsequently cultured for 3 days. Patients P10–P15 had a mean viral load of 18 500 HIV-1 RNA copies (ml plasma)]⁻¹ and a mean CD4 count of 350 cells mm⁻³.

Reverse transcriptase (RT) assays. Culture supernatants (1 ml) were clarified by centrifugation (50 000 g for 90 min using a Beckman J21 rotor) through a Millipore membrane (HAWP, 0.45 µm) and stored at −80°C until the assay. RT activity was measured as described previously (Poli et al., 1990). Briefly, virus pellets were resuspended in 20 µl 50 mM Tris/HCl (pH 7.5), 0.3 M KCl, 1.4 mM dithiothreitol, 15 µg polyadenyl acid ml⁻¹, 15 mg oligothymidylic acid [d(pT)₁₂₋₁₈] ml⁻¹ and 3 mM (111 MBq) [³H]dTMP. After incubation for 1 h at 37°C, the reaction was stopped by addition of 1 ml 5% trichloroacetic acid containing 0.1 mM sodium pyrophosphate, 0.25 ml yeast RNA (0.5 mg ml⁻¹). The material was then incubated in an ice-cold solution of 20% trichloroacetic acid and the incorporated radioactivity was measured as trichloroacetic acid-precipitable material in counting vials containing scintillation fluid. Results were expressed as counts min⁻¹ (ml culture medium)⁻¹.

p24 ELISA. The production of p24 antigen was measured by ELISA. Tissue-culture supernatants were harvested and stored at −70°C until analysis. The commercial kit used was from Abbott.

Statistical analysis. Statistical analysis was done using the Mann–Whitney test for the comparisons of means. P-values <0.05 were considered significant.

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