Human immunodeficiency virus type 1 envelope glycoprotein gp120-mediated killing of human haematopoietic progenitors (CD34+ cells)

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The effects of human immunodeficiency virus type 1 (HIV-1) and recombinant envelope glycoprotein gp120 on the in vitro growth of enriched human haematopoietic progenitors (CD34+ cells) have been investigated. A 2 h exposure to HIV-1 resulted in a progressive and significant reduction of viable CD34+ cell number in liquid cultures and of granulocyte-macrophage, erythroid and megakaryocytic progenitors in semisolid cultures. In virus-treated CD34+ cells, no signs of active virus replication were observed and the possibility of latent infection was excluded by quantitative polymerase chain reaction. Recombinant HIV-1 envelope glycoprotein gp120 added to CD34+ cell cultures displayed a dose-dependent inhibitory activity on CD34+ cell viability. Neutralizing antibody against gp120 was able to block completely the inhibitory activity on CD34+ cells of either HIV-1 or recombinant gp120. These results demonstrate that HIV-1 envelope glycoprotein gp120 has a direct cytotoxic effect on CD34+ cells.

Haematological abnormalities, such as neutropenia, thrombocytopenia and anaemia, are a common finding in human immunodeficiency virus type 1 (HIV-1)-infected individuals (Spivak et al., 1983, 1984; Treacy et al., 1987; Zon et al., 1987; Scadden et al., 1989). Moreover, myelodysplastic changes have frequently been reported in the early stages of the disease, and hypoplasia and fibrosis are characteristically found in the bone marrow in the final period of overt AIDS (Lake et al., 1984; Schneider & Picker, 1985).

Since the earliest description of AIDS-associated haematological abnormalities and myelodysplasia, many studies have been performed to determine how HIV-1 affects, either directly or indirectly, the observed pathology. Studies indicate that, in HIV-1-seropositive individuals, the in vitro growth of bone marrow (Carlo Stella et al., 1987; Leiderman et al., 1987; Ganser, 1988) or peripheral blood haematopoietic progenitors (Bagnara et al., 1991) is often impaired. However many discrepancies among reports on the susceptibility and permissiveness of CD34+ cells to HIV-1 infection, either in vivo or in vitro, still hinder our understanding of the pathophysiology of HIV-1-associated bone marrow and peripheral blood abnormalities.

In vitro infection of haematopoietic precursors, with both monocytotropic and lymphocytotropic HIV-1 strains, has been reported (Folks et al., 1988; Steinberg et al., 1991; Kitano et al., 1991). These results, however, in most instances derived from long-term in vitro experiments, showed that the signs of productive HIV-1 infection paralleled the differentiation of CD34+/CD4- precursor cells towards mature CD34-/CD4+ monocytes and, in some instances, it was not possible to exclude the presence of contaminating CD4+ lymphocytes in CD34+ cell preparations and/or of contaminating provirus DNA in the viral inoculum. On the other hand, in vivo studies have shown that CD34+ cells, purified from the bone marrow of HIV-1-seropositive subjects, only in rare instances present barely detectable signs of latent HIV-1 infection (Von Laer et al., 1990; Molina et al., 1990; Davis et al., 1991), represented by HIV-I provirus DNA sequences demonstrable after polymerase chain reaction (PCR) amplification.

In order to contribute to the understanding of the possible interactions between HIV-1 and human haematopoietic progenitors, we have studied the effect of HIV-1 on the in vitro growth of normal purified CD34+ cells, in short-term experiments.

Bone marrow samples were taken from the posterior iliac crest of 38 healthy donors, after informed consent was obtained. Low density mononuclear cells were isolated by Ficoll–Hystopaque density centrifugation. After two washings in Iscove's modified Dulbecco's medium (IMDM, Gibco) plus 10% foetal calf serum (FCS), mononuclear adherent cells were removed by two successive steps of 1 h adherence in plastic dishes in IMDM plus 10% FCS. Five $\times$ 10^6 to 6 $\times$ 10^6 mononuclear non-adherent cells were pelleted in round-bottomed tubes and 50 µl samples of the following mouse monoclonal antibodies (MAbs) were added to each
pellet: anti-CD2, anti-CD4, anti-CD8, anti-CD11, anti-CD19 and anti-CD20 (Becton-Dickinson) in the presence of 1% bovine serum albumin (BSA, fraction V Cohn, Sigma). After 1 h in ice under continuous agitation, cells were washed twice in order to eliminate the excess MAbs and pelleted. Fifty \( \times 10^6 \) immunomagnetic beads, coated with anti-mouse IgG (MPC 450 Dynabeads, Dynal) were then added to each pellet to obtain an immunomagnetic bead: target cell ratio of 10:1 in a final volume of 0.4 ml for 30 min in ice, under continuous agitation. CD2+, CD4+, CD8+, CD11+, CD19+ and CD20+ cells were removed by the use of a magnet (MPC1, Dynabeads) and the remaining cells were pelleted in round-bottomed tubes (5 \( \times \) 106 cells/tube). CD34+ cells were then positively selected: 50 \( \mu \)l of anti-CD34 (Tecnogenetics) per 5 \( \times \) 106 cells was added to each pellet in a final volume of 150 to 200 \( \mu \)l of PBS with 1% BSA for 1 h in ice under continuous agitation. After two washings, cells were treated with immunomagnetic beads coated with anti-mouse Ig (MPC 450) for 30 min in ice, utilizing an immunomagnetic bead:cell ratio of 3:1. CD34+ cells were collected with a magnet (MPC1) and resuspended in IMDM + 10% FCS. After overnight incubation, CD34+ cells were washed and gently pipetted to facilitate cell separation from the immunomagnetic beads. The final recovery of CD34+ cells ranged from 70 to 80% of the total CD34+ population. The fractional loss was caused by cells dying during the overnight incubation in the absence of growth factors. This enriched CD34+ cell population showed, with immunofluorescent staining, an undetectable reactivity (constantly under 1%) to CD4, CD8, CD11, CD19 and CD20 (Becton-Dickinson).

A lymphocytotropic strain of HIV-1 (HIV \(_{\text{HIV}}\)) was selected, taking into account that CD4+ T lymphocytes are the main HIV-1 reservoir, not only in peripheral blood but also in bone marrow (Davis et al., 1991), and that most patients are naturally infected with lymphocytotropic HIV-1 strains. HIV \(_{\text{HIV}}\) viral stock was represented by an H9 HIV-1-infected (Mann et al., 1989) cell supernatant, clarified by low speed centrifugation, with a reverse transcriptase activity of 5 \( \times \) 105 c.p.m./ml determined as previously described (Re et al., 1989), and an HIV-1 p24 core antigen content of 562 ng/ml determined in a solid-phase sandwich-type capture enzyme immunoassay (DuPont De Nemours). One infectious unit (TCID\(_{50}\)) determined as a syncytium-forming unit by the standard limiting dilution method (0-5 log\(_{10}\) ratio, four replicates per dilution) in C8166 cell cultures (Dianzani et al., 1988) corresponded, in our experimental conditions, to 2-3 log\(_{10}\) of reverse transcriptase activity (c.p.m./ml). CD34+ cells were inoculated with 1 ml of HIV \(_{\text{HIV}}\) (5000 infectious units/10\(^6\) cells) for 2 h at 37 °C. After five washings, 3-5 \( \times \) 10\(^5\) cells were resuspended in 1 ml of IMDM plus 10% FCS plus 2 ng/ml of recombinant human interleukin-3 (rIL3) and incubated in duplicate at 37 °C in 24-well plates (Costar) up to a maximum of 7 days. Viable cells were counted daily by means of trypan blue dye exclusion and p24 was determined in clarified supernatants. Control cultures were run in parallel, treating CD34+ cells with supernatant harvested from mock-infected H9 cells, kept in exactly the same way (cellular density, addition of fresh medium, incubation conditions, etc.) as H9 HIV-1-infected cells. Mycoplasma contamination was excluded in infected and mock-infected H9 cells following the method described by Kaplan et al. (1984).

Liquid cultures of purified human haematopoietic precursors (CD34+ cells) exhibited a significant (\( P < 0.05 \)) and progressive decrease of the number of viable cells with respect to control cell cultures mock-treated with uninfected H9 cell supernatant (Fig. 1). The same cultures were analysed daily, in semisolid medium, to quantify the number of clonogenic cells. Granulocyte-macrophage (CFU-GM), megakaryocytic (CFU-MK) and erythroid (BFU-E) progenitors were assayed in agar, plasmaclot and methylcellulose, respectively, as previously described (Bagnara et al., 1990). As standard sources of colony-stimulating activity, 2 ng/ml of recombinant human granulomacrophage colony stimulating factor (rGM-CSF, Genzyme) for CFU-MK growth, 2 ng/ml rGM-CSF plus 2 ng/ml of rIL-3 (Genzyme) for CFU-GM growth and 2 ng/ml of rIL3 plus 2 units of recombinant human erythropoietin (rEpo, CILAG) for BFU-E growth, were employed. HIV-1-treated cells showed a constantly and significantly reduced number of the different committed haematopoietic progenitors. CFU-GM were the most affected, with a significant difference between HIV-1-treated and
mock-treated controls being apparent after only 2 days of liquid culture (Fig. 2).

No signs of either productive or latent infection were ever observed in HIV-1-treated cells. The total (present in supernatant and cell-associated) amount of HIV-1 p24 core antigen, checked daily in HIV-1-treated CD34+ cell liquid cultures, did not show any increase with time over the background of 0-10 to 0-14 ng/ml, observed at time 0, immediately after washing out the viral inoculum. Moreover no infectious virus was demonstrated in HIV-1-treated cells, either from CD34+ cells in liquid cultures (after 2, 4 and 6 days) or from derived erythroid and granulomacrophage colonies (after 7, 10 and 14 days) assayed by co-cultivation with normal peripheral blood lymphocytes, in the presence of 1 µg/ml of phytohaemagglutinin (Sigma) and p24 determination in co-culture supernatants up to 40 days of culture.

To ascertain whether the impaired in vitro growth of CD34+ cells was due to a latent infection of hematopoietic progenitors by HIV-1, aliquots of 5 x 10⁴ CD34+ cells were tested by PCR for detection of HIV env and/or gag DNA sequences. Cells were washed twice in PBS and digested with proteinase K (60 µg/ml) in 0·45% Tween-20 plus 5 mM-Tris-HCl pH 8·3 for 60 min at 56°C. Subsequently, proteinase K was inactivated by heating at 95 to 100 °C for 5 min and samples were stored at 4 °C until use. The PCR was performed as described by Davis et al. (1991) in a final volume of 50 µl of buffer (10 mM-Tris-HCl pH 8·3, 50 mM-KCl, 2·5 mM-MgCl₂, 0·01% gelatin) containing all four deoxynucleoside triphosphates (0·2 mM each) and 1·25 units of Taq polymerase (Perkin-Elmer Cetus). As a control for amplification efficiency, human leukocyte antigen DQα (HLA-DQα) DNA sequences were co-amplified with env DNA sequences.

For simultaneous amplification of HIV-1 env and cellular HLA-DQα sequences, PCR mixtures contained 50 picomoles each of the env-specific primers SK68 and SK69 (Ou et al., 1988) and 20 picomoles each of the HLA-DQα-specific primers GH26 and GH27 (Scharf et al., 1986). For amplification of the HIV-1 gag sequence, 25 picomoles each of the SK38 and SK39 primers (Ou et al., 1988) were used. PCR amplification was performed for 35 cycles. At the end of the amplification reaction, 20 µl aliquots of the reaction mixture were hybridized in liquid with 32P-labelled probes, represented by SK70 (env), SK19 (gag) or GH64 (HLA). The hybridized samples were subjected to PAGE and subsequent autoradiography at -70 °C. The PCR runs included several reactions containing all reagents except DNA as negative controls, as well as a dilution series of HIV-1 and HLA controls. HIV-1 standards were obtained mixing 2, 10 and 50 8E5/LAV cells, containing a single integrated copy of HIV-1 proviral DNA per cell (Folks, 1986), with 10³ normal peripheral blood leukocytes (PBLs). HLA standards consisted of 10³, 10⁴ and 10⁵ normal PBLs. HIV-1 and HLA controls exhibited a good dose responsiveness of env, gag or HLA signals. The HIV-1 signal was successfully detected in 50% of samples containing two copies of provirus per 10³ cells and in 100% of samples containing 10 and 50 copies of provirus per 10³ cells. Assuming a Poisson distribution for HIV-1 proviral copies in the cellular population under study, these results are consistent with a detection
sensitivity of two or three proviral copies in a background of $10^3$ cells.

Of 12 CD34+ samples, harvested at time 0 and after 24, 48 and 96 h of liquid culture, only one sample out of three, harvested at time 0, showed a weak positive indication of emv DNA sequences, equivalent approximately to two or three HIV-1 provirus copies/$10^3$ cells, probably corresponding to contaminating provirus DNA present in the residual virus inoculum.

The absence of signs of either productive or latent virus infection in HIV-1-treated CD34+ cell cultures suggested that the simple interaction of HIV-1 envelope glycoproteins with cell membranes could be responsible for the cytotoxic effect of HIV-1 on CD34+ cells. Therefore, we studied the influence of the HIV-1 envelope gp120 on the viability of CD34+ cells in liquid culture.

Full-length recombinant gp120 was purchased from MicroGeneSys. It was produced in insect cells using baculovirus expression vectors. Its purity and immunological specificity were further analysed in our laboratory by Western blotting and the preparation was found to be free of bacterial contaminants and, in particular, of endotoxin (Limulus Amebocyte Lysate Test; Whittakers M.A. Bioproducts). CD34+ cells were seeded at a concentration of $25 \times 10^4$/ml in IMDM plus FCS (10%) plus 2 ng/ml rIL-3 in the presence of 0.01 to 10 g/ml of recombinant gp120. A count of viable cells by means of Trypan blue dye exclusion was performed daily up to day 6 of culture.

As shown in Table 1, increasing concentrations of recombinant gp120 (from 0.01 to 10 g/ml) significantly reduced, and in a dose-dependent way, the number of viable CD34+ cells, starting from day 2 of culture ($P < 0.05$), with a maximum cell viability decrease of 70 to 75% reached after 6 days, with the higher concentrations investigated (1 to 10 g/ml). To obtain such inhibitory activity, a short-term (2 h) exposure to gp 120 was not sufficient (data not shown) and the continuous presence of gp 120 in liquid culture was necessary.

### Table 1. Effect of increasing concentrations of recombinant gp120 on CD34+ cell viability

<table>
<thead>
<tr>
<th>Liquid culture (days)</th>
<th>Number* of viable CD34+ cells ($\times 10^3$)</th>
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<tr>
<td></td>
<td>2</td>
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<tr>
<td>Medium alone</td>
<td>247 ± 39</td>
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<tr>
<td>gp120 0.01 g/ml</td>
<td>240 ± 33</td>
</tr>
<tr>
<td>0.1 g/ml</td>
<td>195 ± 31</td>
</tr>
<tr>
<td>1 g/ml</td>
<td>182 ± 24</td>
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<tr>
<td>10 g/ml</td>
<td>185 ± 28</td>
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* Mean values of 10 separate experiments.
† Statistically significant differences between control (medium alone) and gp120-treated cultures.

In further experiments, HIV-1 (5000 infectious units/ml of HIV1) and recombinant gp120 (1 μg/ml) were incubated (1 h at 37 °C) with decreasing amounts (from 50 to 0.1 μg/ml) of a neutralizing anti-gp120 MAb (MAb 9284, DuPont) before the addition of CD34+ cells. A dose-dependent neutralization of the inhibitory effect of both virus inoculum and gp120 on the viability of CD34+ cells was observed (Fig. 3), with its complete inhibition at antibody concentrations from 2 to 50 μg/ml.

The effect of HIV-1 on human haematopoietic progenitors is a controversial topic which continues to loom large in the pathophysiology of HIV-1-associated bone marrow and peripheral blood abnormalities (Folks, 1991).

In this study we have shown that a 2 h exposure to HIV-1 resulted in a progressive and significant reduction of viable CD34+ cells in liquid culture and of CFU-GM, CFU-MK and BFU-E growth in semisolid cultures, in the absence of signs of either productive or latent virus infection. The same results were obtained in CD34+ cell liquid cultures in the presence of purified HIV-1 envelope glycoprotein gp120 alone. The analogy of the mechanisms underlying the cytotoxic effects displayed by either the entire viral inoculum or isolated gp120 was further substantiated by the dose-dependent inhibition of both HIV-1 and gp120 cytotoxic activities, obtained with increasing amounts of a neutralizing anti-gp120 MAb.

Further studies are necessary to identify the CD34+ cell membrane structure(s) with which gp120 interacts and to elucidate the biochemical mechanism(s) of its cytotoxicity for CD34+ cells. However, the results obtained so far clearly point to a direct cytotoxic activity of HIV-1 envelope glycoprotein (gp120) for human
haematopoietic progenitors (CD34+ cells) maintained in in vitro cultures and, although their significance to the understanding of in vivo pathophysiology requires further investigation, they suggest a cautious approach should be taken to the use of massive inoculation of gp120 for vaccination purposes.

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References


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