CD34⁺ Hematopoietic Progenitor Cells Are Not a Major Reservoir of the Human Immunodeficiency Virus

By Dorothee von Laer, Frank T. Hufert, Thomas E. Fenner, Stephan Schwander, Manfred Dietrich, Herbert Schmitz, and Peter Kern

Hematologic abnormalities occur in the majority of patients with acquired immunodeficiency syndrome (AIDS). Infection of the hematopoietic progenitor cells has been proposed as a potential explanation. In this study, different bone marrow cell populations, including the CD34⁺ hematopoietic progenitor cells, were purified by a fluorescence-activated cell sorter (FACS) and analyzed for the presence of human immunodeficiency virus-1 (HIV-1) proviral DNA using the polymerase chain reaction. A group of 14 patients with AIDS or AIDS-related complex (ARC) was studied (11 with peripheral blood cytopenias). The CD4⁺ helper cells in the bone marrow were found positive for HIV-1 DNA in all patients. In contrast, CD34⁺ progenitor cells were positive in only one patient. Two monocytic samples and two samples of CD4⁻/CD34⁻ lymphocytes/blasts (mainly B and CD8 lymphocytes) were positive. Proviral DNA could not be detected in granulocytes. FACS analysis showed that the percentage of CD34⁺ hematopoietic progenitor cells was not altered in the bone marrow of AIDS patients in comparison with the HIV-1 seronegative controls. In contrast, the number of CD4⁺ lymphocytes was markedly reduced in the bone marrow of AIDS patients. These results show that the hematologic abnormalities in AIDS patients are neither explained by direct infection of the hematopoietic progenitor cells with HIV-1 nor by a depletion of progenitor cells.

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nuclear cells (MNC) were washed twice in PBS and subjected to plastic adherence (1.5-hour incubation at 37°C in Iscove's modified Dulbecco's medium (GIBCO, Paisley, UK) containing 25% fetal calf serum (FCS). Cells \((1 \times 10^7/\text{mL})\) were incubated with the particular monoclonal antibodies (MoAbs) for 15 minutes at 4°C. Adherent cells were stained with anti-Leu M3 (CD14)-phycoerythrin (PE) and anti-leukocyte (CD45)-fluorescein isothiocyanate (FITC), while nonadherent peripheral blood cells were stained with anti-Leu 3a FITC and anti-Leu 2a PE (all antibodies from Becton Dickinson, BD, San Jose, CA). Bone marrow nonadherent cells were incubated with a 1:2 dilution of the MoAb anti-HPCA-1 (anti-CD34) for 15-minutes at 4°C, washed twice, and then incubated for 15 minutes with antimouse x PE. Cells were again washed twice and then incubated with 10 vol% mouse serum in PBS for 5 minutes, washed, and stained with Leu3a FITC for another 15 minutes. All cells were fixed for 8 minutes in lysing buffer (BD) after staining, washed twice in PBS, and adjusted to \(2 \times 10^6 / \text{cells/mL}\). Cells were sorted on a FACStar plus cell sorter equipped for four-parameter analysis. A 2-W Argon Laser was operated at 205 mW for the 488-nm line to excite FITC and PE. Analysis and sorting rates were 2,000 cells/min. The phenotypic profile of the sorted cells showed an average purity of CD4+ and CD8+ lymphocytes of greater than 99% with a contamination of less than 1% of CD8+ and CD4+ cells, respectively. CD14+ monocytes/macrophages were recovered in a

![Image of scatter plots showing CD4 and CD34 expression in total and sorted populations.](image-url)

**Fig 1.** Bone marrow mononuclear cells (BMMC) were stained with anti-HPCA-1 (CD34) and anti-Leu3a (CD4), and then sorted on a FACStar as described in Materials and Methods. The total BMMC (10,000 cells) and the sorted cell populations (5,000 cells) were analyzed by FACS. Density blots are shown. Levels: 1, 10; 2, 32; 3, 64; 4, 76; 5, 98; 6, 120; 7, 142; 8, 164; 9, 186.
purity of 98%. The phenotypic profile of the different sorted bone
marrow cell populations is shown in Fig 1. CD34+ bone marrow cells
were obtained in an average purity of 88% with a contamination of
CD4+ cells less than 1%. The sorted CD4+ cells, granulocytes, and
CD4-/CD34- lymphocytes/blasts from the bone marrow samples
reached 96% purity. The contamination with CD4+ lymphocytes of
the latter two was less than 1%.

**DNA Preparation**

**Whole blood.** From 20-mL Na-citrate blood, nuclei were ex-
tracted with NEB (0.3 mol/L sucrose, 10 mmol/L Tris, 5 mmol/L
MgCl2 1% Triton X-100, pH 7.6) and digested with proteinase K (1
mg/mL) in 2.5 mL Lysis buffer (10 mmol/L Tris, 10 mmol/L
Na-EDTA, 50 mmol/L NaCl, 0.5% sodium dodecyl sulfate, pH 7.6)
for 2 hours at 60°C. After digestion, 1 mL of 6 mol/L NaCl solution
was added, the sample spun down, and the supernatant was
precipitated with ethanol and dissolved in TE-buffer
(10 mmol/L Tris, 0.1 mmol/L Na, EDTA, pH 7.4).

**Purified cells.** The different mononuclear cells were washed in
PBS, and 1 x 10^5 to 1 x 10^6 cells were resuspended in 500 μL Lysis
buffer supplemented with proteinase K (1 mg/mL). After an
incubation period of 2 hours at 60°C the proteinase K was inacti-
vated at 95°C (10 minutes) and the crude DNA extract was used for
PCR.

**Synthesis and Purification of the Oligonucleotides**

The oligonucleotides were synthesized using the β-cyanoethyl
method on an Applied Biosystems 381A DNA-synthesizer. The
purification was performed by high-performance liquid chromatogra-
phy using a reverse-phase column (Aqualopore 300, AB7, Weiter-
stadt, FRG; 10 mmol/L Tris HCl, 50 mmol/L KCl, 2.5 mmol/L
MgCl2, 1 mg/mL Gelatine, 0.4% NP40, 0.45% Tween 20, pH 8.3).

**DNA Amplification**

Fifty microliters of the DNA extract were used for amplification.
The samples were amplified using different highly conserved regions
of the HIV-genome. The primer pair SK 38/39 with SK19 as probe
corresponding to the gag region and the primer pair SK 68/69 with
SK 70 as probe corresponding to the env region were chosen for our
experiments.16

Amplification was performed within 35 cycles of PCR using an
amplification mix containing 50 pmol of each primer and 200
μmol/L of each dATP, dCTP, dGTP, dTTP in 10 mmol/L Tris (pH
8.3), supplemented with 50 mmol/L KCl, 2.5 mmol/L MgCl2 and
0.02% gelatin. Two units of Thermus aquaticus polymerase (Perkin-
Elmer Cetus, Emeryville, CA) were added to the final volume of 100
μL. All samples were covered with 2 drops of mineral oil (Sigma, St.
Louis, MO). The cycles started with the denaturation at 95°C for 30
seconds, followed by the annealing at 56°C for 30 seconds, and
finally the elongation at 60°C for 120 seconds. The specific amplified
DNA fragment was detected by liquid hybridization with a 32P
end-labeled oligonucleotide. The hybridization was performed using
10 μL of the amplified DNA sample and 2 x 10^5 cpm of the labeled
oligonucleotide complementary to one of the amplified strands in
0.15 mol/L NaCl solution at 56°C for 30 minutes. The samples were
loaded onto a 20% polyacrylamide gel (mini electrophoresis cham-
ber; Biorad, Munich, FRG) and electrophoresis was run for 25
minutes. The hybrid molecule between the labeled probe and the
amplified target was detected by exposure on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 3 hours at -70°C.

**Controls Used for PCR**

In all amplification experiments, 2 µg DNA both from infected and from uninfected H9 cells were used as a positive and a negative control, respectively. To prove that the reagents were not contaminated with HIV-1 DNA, a TE-buffer control containing no sample DNA was also run in every amplification experiment. Also, different rooms were used for the handling of the samples and for the amplification and preparation of the primers and PCR buffers.

**RESULTS**

The majority of our patients suffered from various blood cytopenias as shown in Table 1. The clinical diagnosis according to the CDC classification ranged from stage II to stage IV HIV disease. Some patients were under antibiotic treatment for toxoplasmosis or *Pneumocystis carinii* pneumonia, explaining to some extent the peripheral cytopenia. In all patients, bone marrow morphology showed a range of myelodysplastic changes typically seen during HIV infection, while in the control group no myelodysplastic changes were observed (data not shown). At the time of analysis, no patient received AZT as antiviral therapy. With the exception of one patient (case no. 4), all patients had low CD4 lymphocyte counts (Table 1).

Bone marrow aspirates were obtained from 14 HIV-1 seropositive and 12 seronegative individuals. The cells were processed and stained with anti–HPCA-1 (CD34) and anti-Leu3a (CD4) as described in Materials and Methods. The phenotypic profile showed that the percentage of CD34+ cells was not significantly different between HIV-1 positive patients and controls. In contrast, the number of CD4+ lymphocytes was reduced in the bone marrows of HIV-1-infected patients (Fig 2).

Various cell populations of blood and bone marrow were tested by PCR for the presence of proviral DNA as described in Materials and Methods (Table 2). Whole blood preparation as well as sorted CD4+ lymphocytes from bone marrow and peripheral blood gave consistently positive results. In serial dilutions of the CD4+ cells, the signal could be detected in at least 1 of 10^3 to 10^4 CD4+ lymphocytes. In contrast, only two blood and two bone marrow monocyte/macrophage samples were positive for proviral DNA. Blood and bone marrow monocytes were never both positive in one individual. With one exception (case no. 8, Tables 1 and 2), the sorted hematopoietic precursor cells of CD34 phenotype turned out to be negative for proviral DNA. Two individuals showed a positive reaction in the CD4+/CD34- lymphocyte/blast bone marrow cell population (case nos. 6 and 11, Tables 1 and 2). CD8+ blood lymphocytes were positive in one case (case no. 11). Proviral DNA could not be detected in bone marrow granulocytes. Although the two primer pairs used in

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**Table 1. Clinical and Hematologic Characteristics of HIV Seropositive Individuals**

<table>
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<tr>
<th>Patient No.</th>
<th>BM Toxic Medication</th>
<th>CDC Stage</th>
<th>Hb g/dL</th>
<th>Pts x 10^9/L</th>
<th>Leuko x 10^9/L</th>
<th>CD4+ Lymph Total %</th>
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<td>II</td>
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<td>340</td>
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<tr>
<td>2</td>
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<td>III</td>
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<td>258</td>
<td>4.9</td>
<td>220</td>
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<td>III</td>
<td>9.7</td>
<td>237</td>
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<tr>
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<tr>
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<td>30</td>
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<tr>
<td>11</td>
<td>+</td>
<td>IV CI</td>
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Some patients were under potentially bone marrow toxic antibiotic treatment for toxoplasmosis or *Pneumocystis carinii* pneumonia (BM toxic medication "+ "). For each patient the CDC stage, the hemoglobin (Hb), the platelet count (Pts), the leukocyte count (Leuko), and the absolute and relative CD4+ cell number as determined by FACS analysis are given.
PROGENITOR CELLS NOT INFECTED WITH HIV

The minimal number of positive cells (mNPC) is given. With SK19 and no reaction on the primer pair from the contaminating CD4+ cells. Cells showing a positive reaction could easily be explained only by proviral DNA in the bone marrow. In support of this argument Folks et al4 found HIV-1 expression in progenitor cells infected in vitro only after these had differentiated towards monocyte/macrophages. However, we have found that the number of CD34+ cells was not reduced in AIDS patients (Fig 2). Furthermore, neither a significant number of monocyte/macrophages nor of the CD4-/CD34- lymphocyte/blast cells contained proviral DNA. Therefore, our results show that the CD4+ cell is the major reservoir of HIV-1 in the bone marrow. Infection of the other cell populations investigated cannot account for the peripheral blood cytopenias in the AIDS patients. Progenitor cells and monocytes/macrophages can be productively infected with HIV-1 in vitro5,19 but in comparison with CD4 cells only serve as a minor reservoir in vivo5,11.

Several possible mechanisms that could lead to peripheral blood cytopenias in AIDS thus remain. Two major cell populations are not covered by our study: megakaryocytes and bone marrow stromal cells. When analyzed by FACS, megakaryocytes show a light scatter pattern different from the other progenitor cells and are not included in the CD34+ cell population studied here. Therefore, our work does not contradict the findings of Zucker-Franklin and Cao,6 who have shown by in situ hybridization that HIV-1 RNA is expressed in megakaryocytes in vivo. This may explain the thrombocytopenias in some AIDS patients. Selective virus infection of bone marrow stromal cells can also affect hematopoiesis. Such a mechanism has been described for the cytomegalovirus.20 Although this possibility should be investigated, several findings indicate that cell populations other than stromal cells are involved in the myelodysplasia seen in AIDS. Stella et al2 have reported that bone marrow mononuclear cells from AIDS patients can regain their proliferative potential after T-cell depletion. Moreover, Leiderman et al21

**Table 2. Detection of HIV-1 Proviral DNA in Different Cell Populations of Blood and Bone Marrow**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CD34</th>
<th>CD14</th>
<th>CD4</th>
<th>CD4+/CD34+</th>
<th>Granulocytes</th>
<th>CD4</th>
<th>CD8</th>
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<td>*</td>
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Different cell populations from blood and bone marrow were purified using a FACS and then analyzed with PCR as described in Materials and Methods. The minimal number of positive cells (mNPC) is given.

*The PCR reaction was negative using DNA from CD4+ cells. NT, not tested.
†PCR was positive with the primer pair from the gag region (probe: SK19) and negative with the env primers (probe: SK70).

**DISCUSSION**

Direct infection of bone marrow progenitor cells has been proposed as a cause for the peripheral blood cytopenias frequently seen in HIV-1-infected patients. In this study, we determined the distribution of HIV-1 proviral DNA in different cell populations of the blood and bone marrow using PCR. Fourteen patients were studied. The CD4+ lymphocyte was found to be the major reservoir of HIV-1 (Table 2). CD4+ helper cells were found positive in all 14 patients, while other cell populations in the bone marrow and blood rarely showed positive results. Only two bone marrow monocyte samples and two blood monocyte samples were positive. In two bone marrow proviral DNA was detected in the CD4+/CD34- lymphocyte/blast population. In only one bone marrow HIV-1 proviral DNA could be detected in CD34+ cells. No viral DNA was detected in granulocytes. In addition, CD4+ lymphocytes had a 10- to 100-fold higher rate of infection than the other positive cell populations. CD4+ cells always reacted with both primer pairs while many of the other cell populations reacted only with the gag primers. Several explanations for these sparse and inconsistent reactions are possible. First, false-positive PCR results can never be completely excluded as this method has an extremely high sensitivity and the purity of the sorted cell populations was not 100% (see Materials and Methods). In case no. 8 at least 1 in 105 CD4 cells and only 1 in 105 CD34 cells were positive. Contaminating CD4+ cells, 0.6%, were found in the sorted CD34+ cell population of this case. Thus, the positive reaction in the CD34+ cells of case no. 8 could easily be explained only by proviral DNA in the contaminating CD4+ cells. Cells showing a positive reaction with SK19 and no reaction on the primer pair from the env region might be infected with mutants altered or even deleted in the env region. It could be argued that HIV-1 proviral DNA cannot be detected in CD34+ progenitor cells because these cells loose the CD34 marker after HIV-1 infection. In this study (SK19, gag, and SK70, env) were equally sensitive for CD4+ lymphocytes, the sparse positive PCR reactions in the other cell populations were obtained partially only with SK19 and not with SK70 from the env region.

Several possible mechanisms that could lead to peripheral blood cytopenias in AIDS thus remain. Two major cell populations are not covered by our study: megakaryocytes and bone marrow stromal cells. When analyzed by FACS, megakaryocytes show a light scatter pattern different from the other progenitor cells and are not included in the CD34+ cell population studied here. Therefore, our work does not contradict the findings of Zucker-Franklin and Cao,6 who have shown by in situ hybridization that HIV-1 RNA is expressed in megakaryocytes in vivo. This may explain the thrombocytopenias in some AIDS patients. Selective virus infection of bone marrow stromal cells can also affect hematopoiesis. Such a mechanism has been described for the cytomegalovirus.20 Although this possibility should be investigated, several findings indicate that cell populations other than stromal cells are involved in the myelodysplasia seen in AIDS. Stella et al2 have reported that bone marrow mononuclear cells from AIDS patients can regain their proliferative potential after T-cell depletion. Moreover, Leiderman et al21...
have found a glycoprotein in the conditioned medium of bone marrow mononuclear cells from AIDS/ARC patients, which inhibits colony growth (CFU-GM) in vitro. In connection with these reports, our data indicate that the high integration of proviral production in show that the percentage of infected progenitor cells and the regulation of blood cell proliferation and differentiation.

Thus, our findings support the use of granulocyte-macrophage colony-stimulating factor (GM-CSF) and other hematopoietic growth factors to overcome the peripheral blood cytopenias complicating late stage HIV-1 infection. In vitro studies show that such factors can enhance virus production in monocytes/macrophages. However, our data show that the percentage of infected progenitor cells and monocytes/macrophages is extremely low compared with the CD4 cells in vivo. Hematopoietic growth factors are not known to affect virus production of CD4 cells, and therefore it is highly unlikely that such factors can cause a substantial increase of the overall virus production in the bone marrow of AIDS patients. However, it will be important to monitor the rate of virus infection of bone marrow monocytes and progenitor cells during clinical studies with G-CSF and GM-CSF.

ACKNOWLEDGMENT

We thank Claudia Juelch, Carola Busch, Annegret Bildhauer, and Claudia Stumme for excellent technical assistance. We thank Dr Ch.J. Hemmer for critical reading of the manuscript.

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CD34+ hematopoietic progenitor cells are not a major reservoir of the human immunodeficiency virus

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