Loss of Primitive Hematopoietic Progenitors in Patients With Human Immunodeficiency Virus Infection

By Aliette Marandin, André Katz, Eric Oksenhendler, Micheline Tuliéz, Françoise Picard, William Vainchenker, and Fawzia Louache

A number of hematologic abnormalities, including cytopenias, have been observed in patients with human immunodeficiency virus (HIV) infection. To elucidate their mechanisms, primitive cells from bone marrow aspirates of 21 patients with HIV-1 infection were quantitated by flow cytometry. The mean percentage of CD34⁺ cells is not significantly altered in HIV-1-infected patients in comparison with HIV-1-seronegative controls. In contrast, two- and three-color immunofluorescence analysis showed that in all HIV-1 samples, most CD34⁺ cells coexpressed the CD38 antigen. The proportion of HIV-1-derived CD34⁺ cells that did not express the CD38 antigen was significantly lower (HIV-1⁺: mean, 1.73%; controls: mean, 14%; P < .0005) than in controls. Moreover, of Thy-1⁺ cells, the proportion of CD34⁺ cells was twofold lower in HIV-1-infected patients (HIV-1⁺: mean, 12%; controls, 25%, P < .0005), which suggests that phenotypically primitive cells are depleted in HIV-1 infection. In vitro functional analysis in long-term cultures of sorted CD34⁺ cells from seven HIV-1 patients showed that CD34⁺ cells from HIV-1 patients generated much fewer colonies both in the nonadherent and adherent layers than CD34⁺ cells from controls after 5 weeks of culture (10-fold and fourfold less, respectively). Precise long-term culture initiating cell (LTC-IC) frequency in the CD34⁺ cell population was determined in three patients by limiting dilution and was markedly decreased in comparison to that of normal controls (from twofold to >7-fold decreased). To determine if primitive cells were infected by HIV-1, both methylcellulose colonies generated from long-term culture of CD34⁺ cells and various CD34⁺ cell fractions purified by flow cytometry were evaluated for the presence of HIV-1 by polymerase chain reaction (PCR). Progeny from long-term culture was HIV-1-negative in three samples. In addition, using a sensitive PCR technique, the HIV-1 genome could not be detected in CD34⁺, CD34⁻/CD38⁻, and CD34⁻/CD38⁺ cells. These data show that hematologic disorders in HIV disease may be the consequence of a deficit of primitive cells. However, direct infection of these cells by HIV-1 does not seem to be responsible for this defect.

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The mechanisms that lead to hematologic abnormalities in human immunodeficiency virus type 1 (HIV-1) infection remain unclear and may be multifactorial. Several nonmutually exclusive hypotheses may be proposed: (1) HIV-1 may directly infect the stem-cell/progenitor compartment and impair their differentiation/proliferation capacities, leading to pancytopenia; (2) HIV-1 may infect regulatory cells, including T cells, macrophages, and stromal cells, and indirectly result in suppression of hematopoiesis; (3) HIV-1 structural or regulatory proteins may suppress hematopoiesis; and (4) antiretroviral medication, antimicrobial therapy, and concurrent viral, fungal, or mycobacterial infection may induce hematopoietic dysfunction.

Conflicting results have been obtained concerning infection of the narrow stem-cell/progenitor compartment by HIV-1. It has been reported that CD34⁺ cells (immature bone marrow cells, including stem cells, progenitors, and precursors) were susceptible to in vitro HIV infection, but that viral replication could only be detected after the induction of a monocytic differentiation. Furthermore, one report suggested that hematopoietic progenitors from HIV-1 patients may express the viral glycoprotein (gp) 120. In contrast, it has been shown by the majority of investigators who used the polymerase chain reaction (PCR) technique that HIV-DNA was not detected either in CD34⁺ cells from HIV-1 patients or in single colonies derived from in vitro differentiation of hematopoietic progenitors.

The use of in vitro clonal assays for multipotent and committed hematopoietic progenitors has shown an impairment in progenitor-cell growth in most studies. Less is known about primitive hematopoietic cells during HIV-1 infection, although one study performed on asymptomatic patients suggested that these cells possess a normal functional capacity. In contrast, another study showed decreased hematopoietic colony growth in long-term cultures of patients with stage IV disease. Recent studies have demonstrated the presence of low levels of functional CD4 receptor on CD34⁺ cells, predominantly on primitive subsets of CD34⁺ cells, which raises the possibility that hematopoietic progenitors may be a target for HIV-1.

In humans, the CD34 antigen is expressed by both committed and primitive hematopoietic stem cells. Other cell-surface antigens, such as the transferrin receptor, CD38, JLA-DR, CD45RA, and Thy-1, have been used to discriminate between immature cells and committed progenitors. Furthermore, the most primitive cells are defined by their capacity to sustain long-term hematopoiesis and to give rise to clonogenic progenitor cells after 4 to 5 weeks of culture on irradiated marrow-derived stroma or competent murine stromal-cell lines. In the present study, we evaluated the compartment of primitive hematopoietic cells in HIV-1-positive patients using both immunophenotyping (CD34⁺/CD38⁻ or CD34⁺/Thy-1⁺) and biologic assays (long-term culture initiating cell assays [LTC-IC]). Moreover, using PCR, we investigated whether these primitive subsets of CD34⁺ cells were infected by HIV-1.

MATERIALS AND METHODS

Patients and Controls

Twenty-one patients with HIV-1 infection were studied. The HIV-1 infection stage diagnosis followed the classification of the Centers...
for Disease Control (CDC; Table 1). Patients were selected based on bone marrow requirement for diagnosis of an associated disease. The mean CD4+ T-cell count was 86 × 10^6 cells/L (range, 0 to 284). Ten of 21 patients had received no antiretroviral therapy or prophylactic antibiotics at the time of study (Table 2). In addition, 10 healthy seronegative individuals without major hematologic abnormalities were investigated as controls.

**Cell Preparation**

After informed consent was obtained from patients and controls, bone marrow cells were collected into a 20-cm³ syringe that contained preservative-free heparin as the anticoagulant. The samples were diluted in Hank's balanced medium (Eurobio, Paris, France) and separated using a Ficoll-metrizoate (Seromed, Berlin, Germany) gradient. Light-density mononuclear cells (LDMC; < 1.077) were washed twice in Hank's medium and used for labeling.

**Antibodies**

Directly conjugated monoclonal antibodies (moAbs) used for flow cytometry were as follows: anti-Leu-3a+3b (SK3+SK4, CD4, fluorescein isothiocyanate [FITC]), HPCA-2 (8G12, CD34, phycoerythrin [PE]), IOA71 (YDJ1.2.2, CD71, FITC), IOB6 (T16, CD38, CD57, 7-ADCC), and 37.4 (CD28, 7-ADCC).

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**Table 1. Main Characteristics of HIV-1-Infected Patient Population**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age</th>
<th>Associated Diseases</th>
<th>CDC Classification</th>
<th>CD4 Cell Count (×10^6/L)</th>
<th>Antiretroviral Therapy</th>
<th>Leukocytes (×10^9/L)</th>
<th>Neutrophils (×10^9/L)</th>
<th>Hemoglobin (g/L)</th>
<th>Platelets (×10^9/L)</th>
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<td>MAI</td>
<td>C3</td>
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<td>No</td>
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<td>7,600</td>
<td>92</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>F/50</td>
<td>NHL</td>
<td>C3</td>
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<td>No</td>
<td>1,000</td>
<td>400</td>
<td>82</td>
<td>24</td>
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<tr>
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<td>CMV</td>
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<td>ddl</td>
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<td>1,500</td>
<td>120</td>
<td>205</td>
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<tr>
<td>4</td>
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<td>ARC</td>
<td>B3</td>
<td>86</td>
<td>AZT</td>
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<td>2,000</td>
<td>130</td>
<td>163</td>
</tr>
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<td>5</td>
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<td>A3</td>
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<td>C3</td>
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<td>63</td>
<td>AZT</td>
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<td>89</td>
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<tr>
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<td>15</td>
<td>AZT</td>
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<td>C3</td>
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<td>No</td>
<td>1,600</td>
<td>900</td>
<td>70</td>
<td>72</td>
</tr>
</tbody>
</table>

**Abbreviations:** MAI, Mycobacterium avium intracellular; NHL, non-Hodgkin's lymphoma; CMV, cytomegalovirus; ARC, AIDS-related complex; PCP, Pneumocystis carinii pneumonia; ITP, immune thrombocytopenic purpura; C meningitis, Cryptococcus neoformans meningitis; KS, Kaposi's sarcoma; MCD, multicentric Castleman's disease; AZT, zidovudine; ddl, didanosine; ddC, zalcitabine; 3TC, lamivudine.

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**Table 2. Clinical Status and Phenotypic Characteristics of CD34+ Cell Population of 10 HIV-1 Patients Who Were Naive to Any Therapy**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Symptoms</th>
<th>CD34*</th>
<th>CD34+/CD38*</th>
<th>CD34+/CD38-</th>
<th>CD34+/Thy-1*</th>
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<tbody>
<tr>
<td>1</td>
<td>MAI</td>
<td>5.5</td>
<td>10</td>
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<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>NHL</td>
<td>6.6</td>
<td>7</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ARC</td>
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<td>8.6</td>
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<td>ITP</td>
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<td>2.4</td>
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<td>8</td>
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</tbody>
</table>

**Mean** 5.1 ± 1.92 10.51 ± 6 1.41 ± 2.1 121 ± 3

**Controls**

|           | 6.5 ± 1.8 (n = 10) | 35 ± 7 (n = 10) | 14 ± 1.7 (n = 10) | 26 ± 3 (n = 6) |

* Percentages of CD34+ cells were determined in lymphoid and blast gate as described in the legend to Fig 1. Percentages of CD34+/CD38* and CD34+/Thy-1* were determined as described in the legends to Fig 2 and Fig 4, respectively, and represent the percentage of CD34+/CD38* or CD34+/Thy-1* among CD34+ cells.

† Significant results (P < .005).

‡ Mean no. ± SD.
FITC), (CD38, R-PE-CYS5), IOT2A (HLA-DR, FITC), IOT4 (13 B 8 2, CD4, FITC), and anti-Thy-1 (5E10, FITC). MoAbs were purchased from Becton Dickinson (Mountain View, CA), Immunotech (Luminy, France), and Pharmingen (San Diego, CA). Red-PE (R-PE)—labeled anti-Leu-3a (SK3, CD4) and HPCA-2 (CD34) were purchased from Becton Dickinson, and OKT4 (CD4) from Coulter (Margency, France). Control isotype antibodies (IgG1, IgG2a, FITC-IgG1, PE-IgG1, and R-PE-Cy5-IgG1) were obtained from Dako (Glostrup, Denmark).

Flow Cytometry and Cell Sorting

Flow cytometry. For single-color immunofluorescence analysis, 1 × 10⁶ LDMC were incubated for 30 minutes at 4°C with PE-conjugated HPCA-2 according to the manufacturer’s instructions. Double- or triple-immunofluorescence analysis was performed using the same procedure and by simultaneously adding one FITC- and one PE-conjugated MoAb, followed by the PE-CYS—conjugated anti-CD38 MoAb.

Cells were suspended in phosphate-buffered saline (PBS), kept at 4°C, and analyzed on a FACScan (Becton Dickinson) using Cellquest software. 5 × 10⁶ events from LDMC and from 2 × 10⁸ to 1 × 10⁹ gated CD34⁺ events were acquired and stored in list mode files.

Cell sorting. For sorting of CD34⁺ cells, marrow cells (10 × 10⁹ cells/mL) were incubated with the PE-HPCA-2 antibody (anti-CD34). Sorting of CD34⁺/CD38⁻ or CD34⁺/CD4⁻ cells was performed by simultaneously incubating the cells with PE-HPCA-2 and FITC-IoB6 (anti-CD38) or FITC-anti-Leu-3a MoAbs (anti-CD4). After one wash, cells were suspended in Iscove’s modified Dulbecco’s medium (IMDM) at a concentration of 2 × 10⁶ cells/mL and separated by cell sorting. Positivity or negativity was determined using control cells labeled with an irrelevant FITC or PE IgG1, PE-IgG1, and R-PE-CYS-IgG1) were obtained from Dako (Glostrup, Denmark).

Assessment of Hematopoietic Progenitor Cells

Quantitation of clonogenic progenitors in semisolid assays. Colony-forming unit—erythroid (CFU-E) and mature and immature burst-forming unit—erythroid (BFU-E), colony-forming unit—granulocyte-macrophage (CFU-GM), and mixed colony-forming unit—granulocyte-erythroid-macrophage-megakaryocyte (CFU-GEMM) progenitors were quantified using previously described methylcellulose culture. Bone marrow cells from each individual were plated at 1 × 10³ cells per well in 24-well plates in the presence of 12.5% horse serum (Hyclone, Logan, UT) and 12.5% FCS (Biochrom, Paris, France). The plates were maintained at 33°C in 5% CO₂ and fed weekly by changing half of the medium. The content of clonogenic progenitors in each well was assessed after 5 weeks in culture by plating 1 × 10⁴ nonadherent cells and 5 × 10⁴ adherent cells (recovered by trypsinization) in methylcellulose (see earlier) supplemented with rhIL-6, rhIL-3, rhEpo, rhSF, and rhG-CSF. For limiting dilution analysis, CD34⁺ cells were directly sorted by the automatic acutocline apparatus of the cell sorter at 20, 50, 100, 200 cells per well in 100 μL medium supplemented with 12.5% horse serum and 12.5% FCS into flat-bottomed 96-well plates seeded with MS-5 cells. For each dilution, a total of 25 wells was prepared. Wells were maintained at 33°C in 5% CO₂ and fed weekly by changing half of the medium. After 5 weeks, cells from each well were recovered by trypsinization, and the content of clonogenic progenitors was assessed in methylcellulose culture. Box—the frequency of positive wells and the content of progenitors per positive well was recorded. A positive well was defined as a well that contained at least one clonogenic progenitor cell after 5 weeks in culture.

Statistics

Results of experimental points obtained from multiple experiments were reported as the mean ± 1 SEM. Significance levels were determined by Student’s paired t test.

PCR

The PCR technique,²⁹ applied to CFU-GM and BFU-E derived progenitors after long-term culture and to sorted CD34⁺ or CD34⁺/CD38⁻, was performed according to the following protocol: sorted cells (1 × 10⁶) or colonies from long-term cultures individually plucked from methylcellulose medium under direct microscopic visualization were transferred directly to amplification microtubes that contained 10 μL 0.9% NaCl solution. A 20-μL reaction mixture that contained PCR buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, and 0.01% gelatin), 0.45% Tween 20, and 400 ng/mL proteinase K was subsequently added. The samples were incubated at 56°C for 50 minutes. Proteinase K was then inactivated by heating at 95°C for 10 minutes. Total extracts were amplified in a final volume of 100 μL containing PCR buffer, 0.2 mmol/L each, and 2.5 μL of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Simultaneous amplification for an HIV-1–specific sequence and GM-CSF was performed by adding 20 pmol of HIV-1 gag-specific amplification primers SK 38/SK 39 and GM-CSF primers composed of a sense primer (5'TTGTACCTC-CAGGAGCCGACC-3') and an antisense primer (5'CTGGTTGGC-ACAGGAACTTGC-3') beginning at positions 943 and 2578, respectively, in the gene sequence of the human GM-CSF gene. The results producting from the clamping of the HIV-1 sequence and GM-CSF were 110 bp and 1,635 bp, respectively. The mixture was overlaid with mineral oil and subjected to 30 repeated rounds of amplification, each consisting of 1 minute of denaturation at 94°C, 1 minute of annealing at 50°C, and 1.5 minutes at 72°C.

Amplification products (10 μL) were resolved on a 2% agarose gel, transferred to Hybond C extra, and hybridized overnight with ³²P-labeled SK 19 and the internal oligonucleotide probe (5'TGCT-TACACAAGCCGGCCTGA-3') for GM-CSF amplification. After washing twice in 2× saline sodium citrate (SSC)/0.1 sodium dodecyl sulfate (SDS) at room temperature and one time in 5× SSC/0.1 SDS at 55°C, the blots were autoradiographed.

The threshold of the PCR technique was set by use of serial dilutions of HIV-infected 8ES/LAV cells.²⁹ Noninfected CEM cells were included as negative control.
RESULTS

Quantitation of CD34+ Cells in Marrow Mononuclear Cells From HIV-1−Infected Patients

Experiments were performed to determine the proportion of CD34+ cells in marrow samples from 21 HIV-1 patients and 10 healthy HIV-1 seronegative controls using direct immunofluorescence analysis. LDMC were stained with the anti–HPCA-2 MoAb (CD34) to determine the proportion of CD34+ cells. This proportion was assessed by two alternative gating procedures defined by the light scatter properties of the cells (FSC and SSC). In the first (Fig IA), CD34+ cells were enumerated in the total low-density (<1.077) cell fraction. In this analysis, a gate was applied to exclude cell debris and mature CS. The mean frequency of cells expressing the CD34 antigen was 3.5% ± 1.8% in controls and 2.4% ± 1.6% in HIV-1 marrow cells (P = .1). The frequency of CD34+ cells was also assessed using a gate based on forward and side scatter, which includes lymphocytes and blast cells, but excludes the majority of monocytic/granulocytic precursors and erythroid cells. The phenotypic profile showed that the percentage of CD34+ cells in this gate is only slightly decreased in HIV-1 samples (Fig 1B).

These results showed that the percentage of CD34+ cells in marrow samples was not significantly different between HIV-1−positive patients and normal subjects.

Subset Analysis of CD34+ Cells

The CD34+ cell population is heterogeneous and includes the whole hierarchy of progenitor cells, as well as some myeloid and lymphoid precursors. To analyze more precisely the number of primitive hematopoietic cells in HIV-1–infected patients, the antigenic profile of CD34+ cells was studied. In an initial set of experiments, the CD38 antigen was investigated, since several studies have shown a substantial enrichment of primitive cells within the CD34+ cell population expressing undetectable or low levels of the CD38 antigen. Furthermore, the level of CD38 expression has been shown to correlate with lineage commitment of CD34+ cells.

Double-staining experiments were performed on LDMC from 21 HIV-1+ samples and 10 controls. Analysis was performed by gating CD34+ cells based on their light scatter properties and high expression of the CD34 antigen. A typical example of this analysis on control marrow is illustrated in Fig 2A through C. As shown in Fig 2B and C, CD38 antigen expression was heterogeneous among CD34+ cells. In control samples, CD34+ cells that are negative for CD38 (CD34+/CD38−) (the threshold of negativity determined by an irrelevant IgG1) were present at a frequency of 14% (n = 10) with remarkable consistency among donors (Fig 2B and Fig 3A). The mean frequency of CD34+ cells that expressed intermediate levels of CD38 antigen, including negative cells (CD34+/CD38−low), was higher (35% ± 7%, n = 10), as shown in Fig 2C and Fig 3B.

In HIV-1 patients, the CD34+/CD38− subset was markedly reduced (1.73% ± 0.5%, P < .0005) in comparison to normal controls (14%) (Fig 2E and Fig 3A). The proportion of CD34+/CD38−low is also decreased as compared with the controls (10% ± 6%, P < .0005) (Fig 2F and Fig 3B).

Thy-1 antigen, which is also restricted to primitive hematopoietic cells, was also used in our study. Two-color analysis was performed on 12 HIV-1 samples and six controls. As shown in Figs 4 and 5, the proportion of CD34+/Thy-1− in HIV-1 samples is twofold lower than in control samples. (HIV-1−median, 12%; range, 10% to 16%; control samples—median, 25%; range, 20% to 28%). This difference was highly significant (P < .0005).

Among the 21 patients enrolled in the study, 11 received...
antiretroviral therapy and 10 were untreated. As shown in Table 2, in all patients evaluated by direct immunofluorescence analysis, the CD34+/CD38−, CD34+/CD38−low, or CD34+/Thy-1− subsets were significantly reduced in comparison to controls, which suggests that antiretroviral therapy did not play a central role in the marked decrease in these two subsets.

These phenotypic results suggested that the proportion of primitive progenitor cells in HIV-1 patients is reduced. To confirm these results, biologic assays were performed.

**Functional Analysis of HIV-1 CD34+ Cells**

The clonogenic progenitor and LTC-IC content of the CD34+ population was assessed in comparative experiments between HIV-1−positive and control samples after cell sorting. Baseline growth (mean ± SEM) of clonogenic progenitors in six HIV-1−positive samples was significantly reduced compared with controls (n = 6) for BFU-E (HIV-1− mean, 30 ± 22; controls, 51 ± 18; P < .025, results expressed per 1,000 plated CD34+ cells) and CFU-GM (HIV-1− mean, 34 ± 17; controls, 57 ± 25, P < .01).

More primitive progenitors were evaluated using the LTC-IC assay. CD34+ cells (5 × 10^5 cells) from seven HIV-1−positive samples and seven controls were cultured in triplicate on the murine stromal cells MS-5. After 5 weeks, nonadherent and adherent cell fractions were evaluated for their clonogenic progenitor content. As shown in Fig 6A, the mean number of clonogenic progenitors from HIV-1 samples (n = 7) in the nonadherent fraction was much lower compared with controls BFU-E (HIV-1− mean, 16 ± 4; controls, 215 ± 43; P < .0005) and CFU-GM (HIV-1− mean, 64 ± 13; controls, 904 ± 148; P < .0005).

Similar results (Fig 6B) were obtained from the adherent cell fraction BFU-E (HIV-1− mean, 8 ± 4; controls, 39 ± 7; P < .001) and CFU-GM (HIV-1− mean, 30 ± 4; controls, 121 ± 30; P < .001).

Our results show that the number of clonogenic progenitor cells present after 5 weeks is significantly reduced in HIV-1−derived CD34+ cells and indicate that LTC-IC frequency is lower in these samples. To measure more precisely the frequency of these cells, limiting dilution analysis was performed. For each evaluation, CD34+ cells were cultured at three cell concentrations in 30 replicate wells. The frequency of negative wells as assessed by the absence of clonogenic progenitors was determined 5 weeks later and LTC-IC frequency in the starting population was calculated using Poisson statistics.

As shown in Fig 7A, in three experiments, the mean fre-
PRIMITIVE CELLS IN HIV' PATIENTS

Fig 3. Summary of FACS analysis of CD38 expression on CD34' cells from controls (n = 10) and HIV-1* (n = 21) samples. Analysis criteria for CD38' and CD38'' were described in Fig 2. Horizontal line represents the mean.

The frequency of LTC-IC was one per 60 CD34' cells from control samples. Using the same procedure, LTC-IC frequency was \( \frac{1}{10^6} \), \( \frac{1}{300} \), and approximately \( \frac{1}{75} \) for the three HIV-1-derived CD34' cell samples, respectively (Fig 7B).

The results obtained by both flow cytometry and functional analysis indicate a severe deficit in primitive cells in acquired immunodeficiency virus (AIDS) patients.

HIV-1 DNA Detection in Primitive Cells

Recent studies have shown expression of CD4 antigen on primitive cells, suggesting that these cells may be targets for HIV. To determine a relationship between the quantitative defect observed in the patients and HIV-1, we first studied whether a selective loss of primitive cells with CD4 antigen expression could be observed. Using two-color experiments, we analyzed CD4 expression on CD34' cells. As shown in a representative experiment (Fig 8A through D), CD4 antigen was detected on HIV-1-derived CD34' cells to the same extent as in controls. Indeed, results between HIV-1 patients (mean, \( 32 \pm 16 \)) and normal controls (31 ± 12) were not significantly different (P > .4). Similar results were found using three different anti-CD4 MoAbs. However, in contrast to normal controls, the CD4 antigen was essentially detected in the CD34+/CD38' cell population by triple-color analysis (data not shown).

To investigate more directly the role of HIV-1 on primitive cells, we used a PCR technique to detect the HIV genome in colonies from long-term marrow cultures. In each of the three patients tested, the HIV genome could not be detected in either BFU-E- or CFU-GM-derived colonies (20 of each) by PCR (data not shown). However, this might indicate that HIV-1-positive LTC-IC are unable to proliferate and differentiate in culture. To exclude this hypothesis, we investigated the presence of HIV-1 gag and env sequences in purified CD34' cells before culture. CD34', CD34'/CD38', and CD34'/CD38'' cells were sorted by means of flow cytometry with a purity greater than 95% from the marrow of five, three, and one patient, respectively. The number of sorted cells ranged from \( 1 \times 10^5 \) to \( 1 \times 10^6 \) for CD34' cells and from \( 3 \times 10^4 \) to \( 5 \times 10^6 \) for CD34'/CD38' and CD34'/CD38'' cells, and was \( 3 \times 10^4 \) for CD34'/CD4' cells. PCR was accomplished on \( 1 \times 10^4 \) sorted cells in duplicate or triplicate depending on the number of sorted cells. To set the threshold of the PCR coamplification, 8E5/LAV cells (containing one HIV-1 genome per cell) were used. Amplification conditions were set up to detect a specific PCR product arising from \( \geq 100 \) 8E5/LAV cells (\( \geq 100 \) HIV-DNA copies), so that contaminating cells would not produce a false-positive result and HIV-1 DNA could not be detected the samples analyzed (including the CD34'/CD38' samples), whereas the internal controls were always positive (Fig 9).

DISCUSSION

Cytopenia and morphologic bone marrow changes are frequently observed in patients with AIDS. However, the mechanisms involved in these cytopenias remain unknown. Recently, it has been shown that a subset of CD34' cells express the CD4 antigen and are able to bind gp120.16,17 A large fraction of these CD34'/CD4' cells correspond to primitive hematopoietic cells both phenotypically (CD34'/CD38' or CD34'/Thy-1+) and biologically, since the majority of LTC-IC are contained in the CD34'/CD4' cell fraction.16 In addition, it has recently been reported that purified blood CD34' cells could be infected in vitro by HIV-1. Therefore, an attractive hypothesis to explain the cytopenia of HIV-1-positive patients might be that HIV-1 infects primitive hematopoietic cells, inducing a progressive loss of stem cells that leads to a major defect in hematopoiesis. The present study was designed to define to what extent primitive progenitors are altered in HIV-1 patients and whether they are infected by HIV-1. Twenty-one patients with an ad-
Advanced disease were investigated, since we thought that such patients were more likely to exhibit a depletion in hematopoietic stem cells than patients studied early in the disease. Initially, CD34+ marrow-cell subpopulations were studied by flow cytometry. Although significantly different, the mean percentage of CD34+ cells was only slightly lower in patients compared with the control group. These results are in agreement with a previous finding that suggested a normal number of CD34+ cells in HIV-1+ patients. Subsequently, we quantitated primitive hematopoietic cells by means of two differentiation markers, the CD38 and Thy-1 antigens. The CD38- subpopulation of CD34+ cells has been shown to include primitive cells, which are highly enriched for blast colony-forming cells and LTC-IC. In contrast, the CD34+/CD38+ cell population contains more committed progenitor cells. Early hematopoietic progenitor cells from fetal and bone marrow are also characterized by the coexpression of the CD34 and Thy-1 antigens. This population has been shown to initiate long-term marrow cultures and to be the CD34 subset that sustains both long-term myeloid and lymphoid human hematopoiesis in immunodeficient mice. In normal human marrow, the CD34+/CD38low and the CD34+/Thy-1+ cell population markedly overlap, since 90% of the Thy-1+ cells are present in the CD38low cell population (our unpublished data). In patients, the CD34+/CD38+ cell fraction was found to be eightfold decreased in comparison to normal controls. The CD34+/CD38low and the CD34+/Thy-1+ were also decreased, but to a lesser extent: 3.5-fold and twofold, respectively. Therefore, there was a slight difference between the expression of the CD38 and the Thy-1 antigens among CD34+ cells in patients. Since CD38 antigen was described as an activation antigen, this difference may be explained by the fact that HIV-1 infection induces an upregulation of the CD38 antigen.
Whatever the reason for this difference, the data showed that during HIV-1 infection, hematopoietic cells with the primitive phenotypes CD34+/CD38− and CD34+/Thy-1− are significantly decreased. However, it cannot be excluded that antigen expression may not entirely correlate with biologic properties, especially in pathologic states. Consequently, a detailed functional analysis was performed using the LTC-IC assay, which is considered to identify a very primitive myeloid progenitor. The overall output of clonogenic progenitor cells among HIV-1–derived CD34+ cells in the LTC-IC assay was fourfold to 12-fold lower than those derived from control samples, which suggests that the LTC-IC frequency was greatly diminished in HIV patients. In three different samples using limiting dilution techniques, this frequency could be directly calculated and was more than two-fold reduced in comparison to normal controls (<1/100 CD34+ cells vs. 1/50 CD34+ cells). Therefore, this result directly parallels those obtained by enumerating the CD34+/CD38low and CD34+/Thy-1− cell population and clearly demonstrates that the compartment of primitive hematopoietic cells is severely depleted in HIV patients with an advanced disease.

If HIV-1 infection is directly responsible for this loss in primitive progenitors, it is expected that the CD34+/CD4− subset that might be infected will be selectively depleted. However, the proportion of CD34+ cells that expressed CD4 antigen was not significantly different from control samples, regardless of the anti-CD4 antibody used. Noteworthy, in contrast to normal controls, CD4 expression among CD34+ cells largely predominates in the CD34+/CD38− cells from the HIV patients. The role of the CD4 antigen in the function of CD34+ cells is presently unknown and this antigen might be involved in functions other than a human leukocyte antigen (HLA) class II recognition, since it has been recently demonstrated to be a cytokine receptor. Therefore, the significance of this change in CD4 antigen expression among
CD34+ subsets in HIV patients remains difficult to understand. Infection of these primitive cells was subsequently investigated by PCR using gag and env primers on individual colonies derived from long-term hematopoietic cultures of three patients. No HIV-1–DNA could be detected in erythroid or myeloid colonies, whereas a control gene was consistently coamplified. These findings are in agreement with several studies that showed an absence of detectable HIV-DNA in CFU-GM– and BFU-E–derived colonies from HIV patients.\textsuperscript{8,10} The presence of HIV genome was also directly tested in the CD34+ cells. This population contains approximately 10% (5% to 20%) progenitor and stem cells.\textsuperscript{25} CD34 cells were sorted with a purity greater than 95%. A PCR technique that allowed the detection of 100 HIV copies was applied on $1 \times 10^4$ cells, and no positive message could be detected. Thus, it appears that the CD34 cells cannot contain more than 1% infected cells. However, it could not be totally excluded that infection occurs in primitive progenitors, which represent less than 1% of CD34+ cells.\textsuperscript{25} To investigate further whether primitive hematopoietic progenitors could be infected, more enriched cell populations (ie, CD34+/CD4+ or CD34+/CD38−) were studied by PCR, but no HIV-DNA could be detected. These data suggest that primitive hematopoietic cells are not infected by HIV-1. Since only patients with advanced disease were evaluated, our studies do not exclude the possibility that primitive cells are infected early in the disease and are subsequently destroyed. Later in the disease, only uninfected primitive progenitors will remain. However, this hypothesis is unlikely, since a recent report has shown a normal number of primitive progenitors and an absence of HIV-1 in CD34+ cells from asymptomatic patients.\textsuperscript{14} Investigation of the expression of the recently discovered HIV-1 coreceptor on primitive hematopoietic cells may allow us to determine whether hematopoietic stem cells can be infected.\textsuperscript{35,37}

Several possible mechanisms may explain this deficit in primitive hematopoietic cells in HIV infection: (1) an alteration in the regulation of hematopoiesis may lead to this defect. Some reports have shown that T cells\textsuperscript{13} or bone marrow mononuclear cells\textsuperscript{38,39} may impair the growth of hematopoietic stem cells.
poietic progenitor by the release of inhibitors. It has also been demonstrated that marrow stromal cells, which play a crucial role in the regulation of early stages of hematopoiesis can also be infected in vitro. Anti-HIV antisenone oligonucleotide treatment or T-cell treatment of HIV-1 marrow leads to a partial correction of hematopoietic progenitor growth. The mediators responsible for this inhibition have not been characterized. However, it has been shown that in HIV-1 infection, CD8 and monocytes release cytokines such as tumor necrosis factor-α, transforming growth factor-β, and several chemokines such as macrophage inflammatory protein-1α, which may specifically inhibit early stages of hematopoiesis; (2) HIV may not infect the CD34+CD4- cell subset, but binding of gp 120 to their surface may trigger programmed cell death as recently suggested; (3) antiretroviral therapy or opportunistic infection may be indirectly responsible for this hematopoietic defect. This hypothesis seems unlikely for antiretroviral therapy, since in this study 10 patients had no treatment at time of in vitro investigation and had a similar decrease in CD34+/CD38− as the other patients. Furthermore, Nea et al observed normal functional properties of CD34+ cells in asymptomatic patients receiving antiretroviral therapy. We cannot totally exclude that opportunistic infection may be partially involved in this defect, since several presently studied patients had such infection. However, a similar defect in CD34+/CD38− cells was found in HIV patients with idiopathic thrombocytopenic purpura; and (4) it is possible that this defect is the consequence of massive destruction of CD4 cells in the periphery and of their continuous replacement. Hematopoietic stem cells give rise to both the myeloid and lymphoid lineages. Thus, it is conceivable that in order to compensate for this consumption of T cells, stem cells preferentially commit towards the T-cell lineage, which leads to a marked deficit in primitive myeloid progenitors. This potential mechanism is supported by the findings in Ikaros knockout mice (which lack a gene that may be involved in lymphocyte commitment), a marked increase in myeloid progenitors is observed. Finally, it is possible that T-cell regeneration may lead to exhaustion of the hematopoietic stem cells compartment. Thus, it will be important in the future to test using both phenotyping analysis and biologic assays and T- and B-cell marrow progenitors in HIV− patients.

In conclusion, this study demonstrates a loss of primitive hematopoietic progenitor cells in HIV patients and strongly suggests that it is not due to a direct infection by HIV. Presently, several investigators have suggested the possibility of an intracellular vaccination for the treatment of HIV-1 infection. This study suggests that, to be feasible, these gene transfer approaches must be performed sufficiently early during HIV-1 disease to target enough hematopoietic stem cells.

ACKNOWLEDGMENT

We thank Drs D. Gillis (Immunex, Seattle, WA) and P. Hunt (Amgen, Thousand Oaks, CA) for the generous gifts of recombinant human IL-3, IL-6, G-CSF, and stem-cell factor. We are grateful to Dr M. Bombard (Hôpital de Villeuneuve, St Georges, France), and Drs Brunet, Laprêle, and Missenard (Clinique Arago, Paris, France) and Koechlin (Hôpital Croix-Rouge, Paris, France) for providing the control bone marrow samples. We thank Dr L. Coulombel for helpful discussions and Dr S. Burstein for improving the English manuscript.

REFERENCES

5. Kitano K, Abbad CN, Ryan DH, Quan SG, Baldwin GC, Golde DW: Macrophage-active colony-stimulating factors enhance human immunodeficiency virus type 1 infection in bone marrow stem cells. Blood 77:1699, 1991
15. Geissler RG, Ottmann OG, Kleiner K, Mentzel U, Bickel-

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Loss of primitive hematopoietic progenitors in patients with human immunodeficiency virus infection

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