Altered Clonogenic Capability and Stromal Cell Function Characterize Bone Marrow of HIV-Infected Subjects with Low CD4+ T Cell Counts Despite Viral Suppression during HAART

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(See the editorial commentary by Badolato on pages 1911–2)

Background. Inflammatory cytokines in bone marrow may impair hematolymphopoiesis in human immunodeficiency virus (HIV)–infected subjects who do not experience reconstitution of CD4+ T cells despite suppression of virus replication while receiving highly active antiretroviral therapy (HAART) (immunological nonresponders).

Methods. Bone marrow samples from 12 immunological nonresponders receiving HAART were studied and compared with samples from 11 immunological responders. The mean CD4+ T cell count (± standard deviation) was 174 ± 68 cells/mm3 and plasma HIV RNA levels had been <50 copies/mL for at least 1 year for individuals enrolled in the study. The clonogenic capability of bone marrow samples was evaluated using the colony forming cell assay and the long-term culture-initiating cell assay. CD34+ cells from the colony forming cell assay were pooled for real-time polymerase chain reaction analysis of Fas and Fas ligand. Bone marrow cytokine production (interleukin-2 and tumor necrosis factor–α) and stromal interleukin-7 levels were analyzed by enzyme-linked immunosorbent assay in both groups. Flow cytometric analysis of CD4+ and CD8+ T cell subsets was performed.

Results. A reduced clonogenic capability and a decrease in the level of more primitive progenitor cells were observed in parallel with lower production of interleukin-2 and increased tumor necrosis factor–α levels. A significant upregulation of Fas and Fas ligand on CD34+ cells and a higher stromal interleukin-7 production were observed. Impairment of the naive T cell compartment and persistent T cell activation were observed in peripheral blood.

Conclusions. Samples from immunological nonresponders show reduced growth of in vitro colonies and an altered cytokine production in bone marrow. The cytokine pattern observed and the altered Fas and Fas ligand pathway may determine stem cell apoptosis and low CD4+ cell recovery. These features, which are similar to those observed in HIV-infected subjects before starting therapy, persist despite treatment.

In most patients who adhere to the treatment, HAART generates a good response, defined by the decrease of plasma viral load to undetectable levels and immunological reconstitution, with a significant increase in CD4+ T cell count [1]. In immunological nonresponders (INRs), it is still unclear why CD4+ T cell counts remain stable, or <200 cells/μL, for >6 months after undetectable HIV RNA levels are achieved [2–4]. In addition, INRs have an impairment of the naive T cell compartment [5, 6]. Immunological, virological, and host-related factors may have an important role in the reconstitution of the immune system. An imbalance of several cytokines, such as IL-7, and abnormalities of the IL-7 receptor have been described in association with CD4+ T cell depletion [7–10]. Many studies have shown that chronic immune activation plays a central role in determining decreases in CD4+ T cell count [6, 11, 12], and during recent years, the role of regulatory...
T cells has gained importance [6, 13]. In AIDS-associated pancytopenia, morphologic bone marrow abnormalities and decreased progenitor cell growth have been described [14]. HIV infection of bone marrow results from the virus’s ability to infect auxiliary cells and alter cell regulatory functions [15, 16]. The depletion of progenitor cells may be related to apoptotic mechanisms, changes in cytokine production, or other immunological factors [17, 18]. Apoptosis of hematopoietic progenitor cells, through the Fas ligand-Fas receptor pathway, is a major mechanism by which activated T cells kill virus-infected cells [19, 20]. Fas ligand and other cytokines produced by activated T lymphocytes and stromal cells contribute to hematopoietic inhibition [21]. We previously examined the effects of antiretroviral therapy on bone marrow activity in HIV-infected patients. HAART induces an improved bone marrow clonogenic capability, which is associated with immunological and hematological reconstitution [22–24]. HAART is also able to promote improvement in stem cell activity [25, 26].

The aim of this study was to investigate the pathogenesis of low CD4+ T cell count in the bone marrow of INRs, in particular focusing on hematopoietic colony formation, the presence of apoptosis in bone marrow cell precursors, and cytokine production of bone marrow cells, including stromal cells.

**PATIENTS AND METHODS**

**Patients.** Twenty-three HIV-infected patients treated with HAART for at least 1 year and followed up as outpatients at the Division of Allergy and Clinical Immunology of the “Sapienza” University of Rome (Rome, Italy) were enrolled in this study. They were selected from a pool of subjects who started receiving antiretroviral therapy during the period from January 2002 through January 2005 and were divided into 2 groups. The first group included 12 subjects who fulfilled the definition of INR (i.e., who had a CD4+ T cell increase of <20% from baseline values and/or a CD4+ T cell level <200 cells/μL and undetectable plasma HIV RNA levels, defined as <50 copies/mL, after 1 year of therapy). In this group, the mean (+ SD) duration of HAART before bone marrow aspirate was obtained was 26 ± 14 months. The second group (virological and immunological responders [IRs]) included 11 subjects with a complete response to therapy after 3–6 months of receiving HAART. The mean (+ SD) CD4+ T cell nadir in the IR group was 105 ± 59 cells/μL (range, 29–240 cells/μL). In the IR group, the mean (+ SD) CD4+ T cell nadir was 155 ± 70 cells/μL (range, 30–277 cells/μL). All enrolled subjects provided written informed consent for the bone marrow aspirate procedure, according to the Ethics Committee procedures of the “Sapienza” University of Rome. Six non–HIV-infected subjects who underwent bone marrow aspirations for peripheral blood hematological abnormalities (with normal results) were included as controls. Table 1 reports the patients’ characteristics.

**Flow-cytometric analysis of PBMCs.** The procedure for whole blood phenotype analysis consisted of lysing 500 μL of blood with 10 mL of Ortho-mune Lysing Reagent (Ortho Diagnostic Systems) at room temperature, washing the sample, and labeling the sample with a cocktail of 4 monoclonal antibodies for 30 min at 4°C. Anti-CD3 fluorescein isothiocyanate, anti-CD4 allophycocyanin, anti-CD8 peridinin chlorophyll protein, anti-CD45RA fluorescein isothiocyanate, anti-CD62L phycoerythrin, and anti-CD19 phycocerythrin were purchased from Becton Dickinson. To identify thymic naive T-helper cells, we used the following antibodies, as described elsewhere [6]: anti-CD4 peridinin chlorophyll protein, CD45RA fluorescein isothiocyanate, and anti-CD31 phycocerythrin, all purchased from Becton Dickinson. After staining, cells were washed once in PBS containing 2% fetal bovine serum and analyzed on a FACSCaliber cytomfluorometer (Becton Dickinson) using Cell Quest software. Absolute lymphocyte counts were calculated by standard hemocytometric technique.

**Viral load determination.** Plasma HIV RNA levels were determined using the Amplicor kit (Roche Molecular Systems). The threshold for measurement was an HIV RNA level of 50 copies/mL.

**Preparation of bone marrow mononuclear cells (BMMCs).** Bone marrow aspirates were collected in a tube with EDTA. The bone marrow samples were diluted 1:3 with 1× PBS plus 5 mmol/L EDTA, separated after centrifugation by Ficoll (Lymphoprep; Nycomed Pharma), and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), and penicillin (250 U/mL) (Life Technologies).

** Colony-forming cell (CFC) assay and long-term bone marrow cultures.** As described elsewhere [22], committed hematopoietic progenitor cells were evaluated in methylcellulose cultures. According to standardized morphologic criteria, the growth of the multipotent hematopoietic progenitor was evaluated as colony-forming unit–granulocyte-erythocyte-megakaryocyte (CFU-GEMM), burst-forming unit–erythroid (BFU-E), CFU-erythroid (CFU-E), and CFU-granulocyte-macrophage (CFU-GM). In addition, the effects of IL-2 on bone marrow colony formation was studied by CFC assay. To analyze the most immature progenitors and the stem cell compartment, we used long-term bone marrow culture. Stromal cell cultures and long-term culture-colony forming cell (ITC-CFC) determinations were performed as described elsewhere [22]. Moreover, the effects of IL-2 (10 ng/mL) addition on bone marrow colony formation by ITC-CFC assay were evaluated in a sample of INRs.

**Analysis of Fas, mFas, and Fas ligand on CD34+ cells by real-time PCR.** Colonies obtained from the methylcellulose assay (CFC assay) of INRs were selected, washed twice in PBS, and pooled for real-time PCR analysis of Fas and Fas ligand (2 × 10^4 cells to 5 × 10^5 cells). Total RNA was obtained using
the RNeasy Minikit (Qiagen), according to the manufacturer’s instructions [27].

**Spontaneous cytokine production from BMMC cultures.** To evaluate cytokine production, BMMC short-term cultures were performed using freshly collected bone marrow samples. In brief, isolated BMMCs were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), and penicillin (250 U/mL) in 5% CO₂ atmosphere at 37°C, in the absence of stimuli, to verify spontaneous production of IL-2 and TNF-α. After 24 h, supernatants were collected, and measurement of cytokines was performed by ELISA, according to the manufacturer’s instructions (R&D System).

**Immunohistochemistry characterization of bone marrow stromal cells.** BMMCs were cultured in tissue culture chamber slides (Falcon) in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO BRL Life Technologies) supplemented with 20% fetal calf serum, 100 IU/mL penicillin-streptomycin, and 100 IU/mL glatamine at 37°C in humidified air with 5% CO₂. At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and the replacement of 500 μL of fresh supplemented IMDM. The cultures were maintained until stromal confluence (3–4 weeks) and then analyzed by immunohistochemistry, as described elsewhere [26]. The slides were analyzed using light microscopy.

**Spontaneous IL-7 production from bone marrow stromal cultures.** BMMCs were cultured in 24-well plates in IMDM supplemented with 10% fetal calf serum, 10% horse serum, 100 IU/mL penicillin-streptomycin, 100 IU/mL glutamine, and 1×10⁻⁸ mol/L hydrocortisone sodium succinate (Sigma) at a concentration of 1×10⁶ cells/mL in a total volume of 2 mL per well. The plates were incubated at 37°C in humidified air with 5% CO₂. At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and the replacement of 500 μL of fresh supplemented IMDM. The cultures were maintained until stromal confluence (3–4 weeks), and then the cells were collected by trypsinization and cultured at a concentration of 1×10⁶ cells/mL in a total volume of 1 mL per well. Supernatants were collected after 24 h, and IL-7 levels were determined using an ultra-sensitive ELISA kit (R&D System).

**Statistical analysis.** Nonparametric statistics were used (Mann-Whitney test and Wilcoxon rank-sum test) for unpaired and paired comparisons between the parameters analyzed in patients and healthy individuals. A simple regression test was used to correlate the group characteristics. A P value <.05 and an r value >.5 or less than −.5 were considered to be statistically significant. Statistical analyses were performed using Stat View software, version 5.0 (SAS Institute).

**Figure 1.** Percentage and absolute count of CD4⁺ T cells (A, B) and naive CD4⁺ T cells (C, D) in immunological nonresponders (INRs), immunological responders (IRs), and the control group (HIVneg).
RESULTS

Flow cytometric analysis of T cell subsets. As described elsewhere [6] and shown in figure 1, measured as both a percentage and an absolute cell count, a significantly lower CD4+ T cell level was present in peripheral blood samples obtained from INRs. Mean values (±SD) were 16.2% ± 9% and 183.7 ± 85.8 cells/µL in the INR group, compared with 85.8 ± 30% (P = .007) and 615.6 ± 159.6 cells/µL (P = .001) in the IR group. Moreover, among CD4+ T cells, the naive compartment was significantly reduced in the INR group versus the IR group (26.3% ± 17.2% and 53.2 ± 48.5 cells/µL vs. 43.1% ± 11.8% (P = .04) and 258 ± 71.8 cells/µL (P = .001), respectively). In addition, INRs demonstrated an increased expression of activation markers (HLA-DR, CD95, and CCR5; data not shown), as reported elsewhere [6]. In contrast, no significant differences among CD8+ T cell subsets were observed between the 2 groups; CD8+ T cell counts were 42.5% ± 13.1% with 561 ± 387 cells/µL in the INR group, compared with 38% ± 13% (P = not significant) with 848 ± 473 cells/µL in the IR group (P = not significant).

CFC assay and long-term bone marrow cultures. In figure 2, results of CFC assays from INRs are shown and compared with those from IRs before and after HAART, as well as with results of CFC assays of non–HIV-infected control subjects. The CFC result was lower for bone marrow obtained from INRs than for bone marrow obtained from IRs after HAART, although the difference was not always statistically significant (11.2 ± 20.9 vs. 21.7 ± 20.3 CFU-E [P = not significant], 45.2 ± 29.3 vs. 80 ± 38.1 BFU-E [P = .04], 3.5 ± 4 vs. 6.4 ± 4.6 CFU-GEMM [P = not significant], and 34.3 ± 14.1 vs. 83.8 ± 42.6 CFU-GM [P = .003].) These values were quite similar to those observed in HIV-infected subjects before starting therapy. The content of primitive progenitors was evaluated by the LTC-CFC assay. The number of primitive bone marrow progenitor cells in INRs showed a tendency (although not statistically significant) toward reduced production, compared with the number of such cells in IRs (5.8 ± 4.5 vs. 8.4 ± 6.3 LTC-CFC/1 × 10^6 BMMCs) and was similar to the number in HIV-infected subjects before starting HAART (data not shown). The addition of IL-2 to the CFC and LTC-CFC assays induced an increased growth of in vitro colonies, especially those of the more-immature progenitor cells (figure 3).

Analysis of Fas, mFas, and Fas ligand on cells from the CFC assay by real-time PCR. A significant upregulation of Fas mRNA (20 ± 21.8 vs. 6.6 ± 5.6 copies/1000 high density lipoprotein–binding protein mRNA [HBP] copies; P = .03) and Fas ligand mRNA (15.2 ± 4.7 vs. 3.7 ± 4.1 copies/1000
Hematological abnormalities frequently occur in HIV-infected patients. Increasing evidence indicates that bone marrow suppression results from viral infection of accessory cells, resulting in impaired stromal function and alteration of the hematopoietic growth factor network. In this study, the pathogenesis of low CD4+ T cell count in INRs at the bone marrow level has been investigated, with particular focus on hematopoietic

DISCUSSION

Hematological abnormalities frequently occur in HIV-infected patients. Increasing evidence indicates that bone marrow suppression results from viral infection of accessory cells, resulting in impaired stromal function and alteration of the hematopoietic growth factor network. In this study, the pathogenesis of low CD4+ T cell count in INRs at the bone marrow level has been investigated, with particular focus on hematopoietic

HBP copies; $P = .03$) was observed in CD34+ cells derived from CFC assays of INRs (data not shown).

**Measurement of spontaneous cytokine production in BMMC cultures.** In vitro production of IL-2 and TNF-α by short-term culture of BMMCs (figure 4A) was determined for samples obtained from INRs and compared with results for samples obtained from IRs. The mean (± SD) amount of IL-2 produced was lower in the INR group than in the IR group (32.3 ± 3 pg/mL vs. 39.5 ± 7 pg/mL; $P$ not significant). In contrast, the mean (± SD) spontaneous production of TNF-α was significantly greater in the INR group than in the IR group (32.2 ± 7 pg/mL vs. 18.7 ± 5 pg/mL; $P = .04$).

**Measurement of spontaneous cytokine production from bone marrow stromal cells.** The stromal cells obtained from INRs spontaneously produced significantly higher mean (± SD) levels of IL-7, compared with those obtained from IRs (0.4 ± 0.04 pg/mL vs. 0.1 ± 0.1 pg/mL; $P = .02$) (figure 4B).

**Morphology and immunohistochemistry of bone marrow stromal cells.** Stromal layers yielded positive results for CD68 and CD14 but negative results for S100 and CD34 molecules, indicating that these cells were of the macrophage-monocyte lineage. In all INRs, the majority of these cells appeared as large, rounded cells with abundant cytoplasm on light microscopic examination, in contrast with those cells observed in IRs, in whom 60%-80% of the stromal cells exhibited a “fibroblast-like” morphology after HAART. These morphologic characteristics are quite similar to those observed in patients before starting antiretroviral treatment (figure 5).

**Figure 3.** Effect of IL-2 on colony-forming cell and long-term culture-initiating cell (LTC-IC) assays in INRs. The bars represent the mean fold increase (FI), compared with the results obtained without growth factors, of colonies obtained by colony-forming cell assays and by LTC-IC assays from 3 INRs with the addition of IL-2 (10 ng/mL). BFU-E, burst-forming unit–erythroid; CFU-E, colony-forming unit–erythroid; CFU-GEMM, colony-forming unit–granulocyte-erythrocyte-monocyte-megakaryocyte; CFU-GM, colony-forming unit–granulocyte-monocyte.

**Figure 4.** Spontaneous IL-2 (A) and TNF-α (B) production in bone marrow mononuclear cell cultures, and spontaneous IL-7 (C) production from bone marrow stromal cells. INR, immunological nonresponder; IR, immunological responder.
colony formation, the presence of apoptosis in bone marrow cell precursors, and the cytokine production of bone marrow cells, including stromal cells, before and during antiretroviral treatment. Compared with non–HIV-infected control subjects, altered cytokine and chemokine production by bone marrow cells was observed before treatment, characterized by decreased IL-2 levels and elevated TNF-α, MIP-1α, MIP-1β, and RANTES levels, along with defective bone marrow clonogenic activity. Antiretroviral therapy was associated with an amelioration of stem cell activity, a restoration of stromal cell pattern and functions, and an increased IL-2 production in bone marrow and a decrease of Fas expression on progenitor cells, in parallel with the diminution of TNF-α levels [25]. Moreover, HIV protease inhibitors may improve hematopoietic functions because of their direct effect on bone marrow progenitor cells [26]. A consistent quantitative and functional hematopoietic progenitor cell defect was observed in INRs, suggesting an impaired clonogenic potential of bone marrow progenitor cells and a decreased progenitor cell recovery in the long-term culture-initiating cell assay. This abnormality was associated with altered stromal cell composition and cytokine production. Such defects may be attributable, at least in part, to increased TNF-α production by inflammatory cells in the bone marrow and to decreased IL-2 levels. IL-2 is a cytokine that stimulates proliferation and function of CD4+ and CD8+ T lymphocytes, B lymphocytes, and natural killer cells in vitro. The positive effects exerted by IL-2 on T cell apoptosis have already been described. In fact, the addition of IL-2 to the lymphocyte cultures reduced apoptosis of PBMCs obtained from patients with secondary immune deficiencies [28]. On the other hand, it is well known that the suppressive effect of TNF-α on bone marrow progenitor cells is mediated by affecting cell viability or by modulating the expression of cytokine receptors on the cell surface [29]. TNF-α is a well-known negative regulator of hematopoiesis which induces functional Fas on CD34+ cells [30, 31] and promotes their differentiation [32]. The high TNF-α levels and the decreased IL-2 production observed in the more primitive compartment might mediate the apoptotic depletion of progenitor cells, as demonstrated by the reduced growth of in vitro colonies. The addition of IL-2 increased the growth of in vitro colonies, suggesting an important role of this cytokine for committed and uncommitted progenitor cells. These results provide support to other possible mechanisms of in vivo action of IL-2 in addition to that reported in several clinical trials that used this cytokine associated with HAART [33–35]. Our data confirm that the naive CD4+ T cell subset is severely damaged in INRs. In fact, we observed a significant reduction of CD4+, naive, and thymic naive T cells compared with IRs. Chronic immune activation plays a central role in determining CD4+ T cell decreases [11, 13] and, as recently reported, the role of

Figure 5. Morphology of bone marrow stromal cells from immunological responders before (A) and after (B) HAART and from immunological nonresponders (C). The stromal cells were sorted onto slides and fixed in an acetone-ethanol (50:50 ratio of acetone:ethanol) solution before staining with anti-CD68. The slides were analyzed by light microscopy (original magnification, ×40).
regulatory T cells in modulating the immune response has gained importance [6, 13]. In addition to the mechanisms described in peripheral blood and in lymph nodes [6, 12], the immunological defect of INRs may be the result of bone marrow impairment. In the same patients, we found a reduced clonogenic potential, including more immature lymphoid progenitor cells, in parallel with the increased expression of Fas and Fas ligand on the stem cells as the result of increased stem cell death by apoptosis. In vivo and in vitro hematopoiesis occurs in association with the complex network of cell types in the stroma. The prevalence of macrophage-like cells in long-term bone marrow culture, rather than the typical “fibroblast-like” cells, suggests the existence of a modified composition of the bone marrow stroma, possibly linked to an underlying inflammatory process within the bone marrow microenvironment. In INRs, all abnormalities described in the bone marrow before starting HAART remained unchanged despite the prolonged control of virus replication in peripheral blood. Bone marrow (CFC and stroma) and peripheral blood findings, described here, confirm that the persistence of an immune activation status (increased production of TNF-α and apoptosis) plays a central role in the pathogenesis of the discordant response to the antiretroviral therapy.

A central function of stromal cells is IL-7 production [36]. Because IL-7 acts as a growth and antiapoptotic factor for B cell and T cell precursors, its production is a critical step for the beginning of lymphopoiesis starting from the stem cells. Compared with the stromal IL-7 production in IRs, we observed elevated IL-7 levels in INRs. In HIV-infected subjects, it has been shown that CD4+ T cell depletion is associated with an increase in IL-7 levels, whereas after antiretroviral therapy, CD4+ T cell counts increase in parallel with decreased IL-7 levels [37]. Elevated IL-7 levels may be related to CD4+ T cell depletion, but we cannot rule out the possibility that IL-7 production is irrelevant to the degree of CD4+ T cell depletion.

In conclusion, a reduced content of in vitro committed and primitive progenitors was observed in BMMCs of INRs and was associated with phenotypic abnormalities of stromal cell composition and cytokine production. These abnormalities may be attributable, at least in part, to the increased TNF-α production by inflammatory cells in the bone marrow and to the observed decrease of IL-2 levels. We hypothesize that the

### Table 1. Demographic, hematological, immunological, and virological characteristics of enrolled subjects at the time of bone marrow aspirate collection.

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<th>Hemoglobin level, g/dL</th>
<th>Platelet count, platelets/μL</th>
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| Immunological nonresponders |
| 1       | F   | 40         | C3            | 2400                 | 15                     | 166,000                    | 237                         |
| 2       | F   | 52         | A3            | 3640                 | 13                     | 205,000                    | 242                         |
| 3       | F   | 46         | C3            | 3280                 | 12                     | 335,000                    | 138                         |
| 4       | M   | 63         | C3            | 3710                 | 13                     | 192,000                    | 228                         |
| 5       | M   | 41         | A3            | 3690                 | 14                     | 115,000                    | 239                         |
| 6       | M   | 41         | A3            | 1590                 | 10                     | 46,000                     | 134                         |
| 7       | M   | 41         | A2            | 7270                 | 13.8                   | 236,000                    | 214                         |
| 8       | M   | 46         | B2            | 3500                 | 15                     | 89,000                     | 232                         |
| 9       | F   | 44         | A3            | 9240                 | 10.9                   | 380,000                    | 186                         |
| 10      | M   | 41         | B3            | 4440                 | 15                     | 126,000                    | 72                          |
| 11      | M   | 48         | C3            | 5530                 | 16.1                   | 149,000                    | 116                         |
| 12      | F   | 36         | A3            | 4140                 | 13.4                   | 182,000                    | 56                          |

**NOTE.** All patients had an HIV RNA level <50 copies/mL.
damage of the T cell compartment may be at least partially attributable to an altered generation of new cells committed to the T lineage from the hematopoietic stem cell and/or progenitor cells. These results give additional support to consideration of the therapeutic administration of IL-2 and the use of antipoptotic factors as a strategy in the treatment of INRs.

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