To define the relationship between human immunodeficiency virus type 1 (HIV-1) infection in hematopoietic stem cells and virus production by their progeny, we performed kinetic studies infecting bone marrow (BM) stem cells and culturing them in the presence of hematopoietic growth factors. CD34-positive (CD34+) and CD4-negative (CD4-) BM cells were isolated and infected in vitro with the monocytotropic HIV-1 \( \text{III}_{\text{a}}, \text{strain, or the laboratory-maintained HTLV-Ill,} \) and virus production as measured by p24 protein release was markedly increased (more than fivefold) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3). Macrophage CSF (M-CSF) was less stimulatory and granulocyte CSF (G-CSF) had no effect on virus production. Virus production coincided with proliferation of mononuclear phagocytes but was not related to granulocytic proliferation in G-CSF-treated BM cultures. Although peak virus production from GM-CSF–treated macrophages occurred 2 to 3 weeks after infection, peak virus production in infected stem cells was observed 5 to 6 weeks after. Enhancement in virus production had a more rapid onset when CD34+/CD4- cells were cultured in the presence of both GM-CSF and IL-3 for 7 or 14 days. Under these conditions there was a 10-fold enhancement in virus production after 7 days of preincubation and a 50-fold enhancement after 14 days. These data indicate that while the stem cell compartment may be susceptible to infection with a monocytotropic HIV-1 strain, productive and sustained infection is realized only after macrophage differentiation. The lack of effect of G-CSF on virus production is likely because of the limited effect of this hematopoietin on mononuclear phagocyte generation and function.

Hematopoietic abnormalities in acquired immunodeficiency syndrome (AIDS) are common and multifactorial. Bone marrow (BM) hematopoietic stem cells can be infected with human immunodeficiency virus type 1 (HIV-1) in vitro and may be targets for infection in vivo. Among the mature blood cells, only T lymphocytes and macrophages are known to be directly infected with HIV-1 and infection of these cells causes immunodeficiency by impairing their proliferative and functional activity. However, it is not certain to what degree infection of stem cells may relate to hematopoietic dysfunction or to ultimate production of virus by mature progeny.

Hematopoietic growth factors are used extensively in clinical trials in HIV-1–infected persons, and granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte CSF (G-CSF), and erythropoietin (Epo) have proved effective in improving the hematologic status of patients with AIDS. The use of hematopoietic growth factors to ameliorate the hematologic toxicity of azidothymidine (AZT) is particularly promising. Because macrophage-active growth factors such as GM-CSF, macrophage CSF (M-CSF), and interleukin-3 (IL-3) are known to increase HIV-1 replication in mononuclear phagocytes in vitro and in perhaps in vivo, their use could have deleterious effects on HIV-1–infected individuals. A similar theoretical concern applies to effects of hematopoietic growth factors on hematopoietic stem cells in the situation where such cells may be targets for HIV-1 infection. We have investigated the effect of GM-CSF, G-CSF, IL-3, and IL-6 on HIV-1 infection in CD34+/CD4- BM cells and subsequent expression of virus in their progeny and found that HIV-1 production in myeloid progenitor cultures was enhanced in the presence of the macrophage-active CSFs GM-CSF, IL-3, and M-CSF. However, G-CSF and IL-6 did not enhance virus production.

MATERIALS AND METHODS

BM cells. BM cells were obtained from healthy volunteers with appropriate written informed consent. Light density cells were separated by Ficoll-Hypaque gradient centrifugation (400g for 30 minutes). The adherent cells were removed by adherence to plastic dishes containing 10^5 cells/mL in Iscove’s modified Dulbecco’s medium (IMDM, Irvine Scientific, Santa Ana, CA) supplemented with 5% human AB serum (Irvine Scientific) and 15% fetal bovine serum (FBS; Gemini Bio-Products Co, Calabasas, CA) incubated at 37°C and 5% CO2 in air for 3 hours. The light density and nonadherent cell fractions were further enriched by T-cell depletion using rosette formation with neuraminidase-treated sheep erythrocytes.

A CD34+/CD4- fraction was sorted from light density BM cells without plastic adherence using anti-CD34 (My10) antibody and anti-CD4 antibody with a sterile two-step flow cytometric technique as previously reported. Briefly, Ficoll-Hypaque–separated BM cells (10^6 cells/200 mL) were stained with a 1:20 dilution of anti-CD34 (IgG1; Becton Dickinson, Mountain View, CA) and a 1:20 dilution of anti-CD4 (IgG2a; Accurate Chemical and Science Corporations, Westbury, NY), then incubated at 4°C for 30 minutes. After washing three times with normal saline solution, cells were stained with 1:20 dilutions of fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG1 antibody (Fisher Scientific Company, Tustin, CA) and phycoerythrin...
conjugated goat antimouse IgG2a antibody. CD34+/CD4- cells were sorted on a Xerox 7020 (Xerox, Rochester, NY) under aseptic conditions at high speed (4,000 to 6,000 cells per second). The cells were centrifuged, resuspended in phosphate-buffered saline (PBS), and sorted again at low speed (500 to 1,000 cells per second). The CD34+/CD4- cell population was judged to be 99% viable by trypan blue exclusion.

HIV-1 strains. Y. Koyanagi and I.S.Y. Chen (UCLA, Los Angeles, CA) provided both a primary monocytotropic HIV-1 isolate, HIV-1 mrl, and a laboratory-maintained strain of HTLV-IIIb, which has little tropism for mononuclear phagocytes.14 HIV-1 mrl, and HTLV-IIIb viruses were propagated in phytohemagglutinin-stimulated peripheral blood lymphocytes.

Hematopoietic growth factors. Purified human recombinant (rhu) GM-CSF, IL-3, M-CSF, and IL-6 were all provided by Genetics Institute, Inc (Cambridge, MA). rhu G-CSF KW-2228 was provided by Kyowa Hakko Kogyo Co, LTD (Japan).30,31

HIV-1 infection of BM cells. Non-Ad/non-T BM cells were infected with HIV-1 mrl, at a concentration of 500 ng of HIV-1 p24 protein per 2 x 10^6 cells (multiplicity of infection [MOI] = 0.05) in the presence of 10 µg/mL Polybrene (Sigma, St Louis, MO) for 2 hours at 37°C. CD34+/CD4- BM cells were infected at a concentration of 500 ng HIV-1 p24 protein per 10^6 cells (MOI = 1). As a negative control, cells were incubated with either heat-inactivated virus or β-propiolactone (Sigma) treated virus.77 After washing three times, the non-Ad/non-T cells or the CD34+/CD4- cells were resuspended in medium at a concentration of 5 x 10^6/mL or 5 x 10^5/mL containing 10% fetal calf serum (FCS) and hematopoietic growth factors.

To assay for productive virus infection, cell-free supernatants were assayed for p24 antigen release using a commercial enzyme immunoassay (Abbott Laboratories, North Chicago, IL). MOI was determined by titration assays: HIV-1 mrl, infecting mononuclear phagocytes; HTLV-IIIb, infecting phytohemagglutinin-stimulated peripheral blood lymphocytes.

Proliferative assays. Test cells (2 x 10^4) were incubated in 0.2 mL IMDM in 96-well flat-bottomed microtiter trays. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. DNA synthesis in the cultures was determined after addition of [3H]-thymidine (0.5 µCi/well) for 24 hours. The cells were recovered on glass fiber filters and the filters were counted in liquid scintillation fluid (Fluorodyne; National Diagnostics, Somerville, NJ).

RESULTS

Kinetic analysis of HIV-1 production and [3H]-thymidine incorporation in nonadherent and T-cell-depleted BM cells. To clarify the relationship between HIV-1 production and cell proliferation in BM cells, a kinetic study of p24 antigen production and [3H]-thymidine incorporation in HIV-1 mrl-exposed non-Ad/non-T cells was performed in the presence or absence of GM-CSF. A rapid increase in virus production was observed after 3 weeks when the cells were differentiated into macrophages, and peak production was observed 6 weeks after infection when the [3H]-thymidine incorporation was decreasing (Fig 1A). In the absence of GM-CSF, slight increases in [3H]-thymidine incorporation, as well as virus production, were noted and peak virus production occurred about 6 weeks after infection (Fig 1B). More than a 100-fold increase in virus production was observed in the presence of GM-CSF as compared with control.

Dose response of GM-CSF and G-CSF on HIV-1 production. GM-CSF stimulated both macrophage and granulocyte proliferation and differentiation in non-Ad/non-T BM light density cells, and virus production was enhanced as the concentration of GM-CSF was increased (Fig 2A). In contrast, G-CSF stimulated only granulocyte proliferation and differentiation and had little, if any, effect on virus production (Fig 2B).

Kinetic studies of HIV-1 production in CD34+ and CD4- BM stem cells. CD34+/CD4- BM cells were sorted using the two-step flow cytometric technique (Fig 3) and then infected with HIV-1 mrl or HTLV-IIIb in the presence of Polybrene. Viral infectivity was monitored by assaying p24 antigen release in the supernatants every 2 days. When the cells were cultured in the presence of a combination of CSFs that included GM-CSF, G-CSF, M-CSF, IL-3, and IL-6, virus production from HIV-1 mrl-infected cells increased rapidly after 10 days of incubation, but no significant virus production was detected in cells exposed to HTLV-IIIb (Fig 4). In the absence of CSFs, virus production was not enhanced. Using heat-inactivated virus particles, nonspecific binding of virus to the cell surface was estimated at less than 30 pg/mL of p24 virus protein.

Fig 1. Kinetic analysis of HIV-1 production and [3H]-thymidine incorporation in HIV-1-infected BM cells. Normal nonadherent, T-cell-depleted, light density BM cells were infected with HIV-1 mrl, at an MOI = 0.05. The cells were cultured in the presence of GM-CSF (A) or in the absence of GM-CSF (B). HIV-1 p24 protein (*) in the supernatants was assayed in duplicate and [3H]-thymidine incorporation (□) was measured in triplicate.
HIV-1 infection of cells of the monocyte/macrophage lineage is believed to be important in the pathogenesis of AIDS and direct infection of mononuclear phagocytes in vitro by monocytotropic HIV-1 strains is routinely accomplished. Recent evidence indicates that purified populations of myeloid progenitor cells are susceptible to HIV-1 infection in vitro as well.

Mycelopoesis is regulated by hematopoietic growth factors, including IL-3, GM-CSF, M-CSF, and G-CSF, and...
both GM-CSF and G-CSF have been used in clinical trials to stimulate myelopoiesis in patients with HIV infection. However, little is known about the effect of hematopoietic growth factors on HIV-1 infection in hematopoietic stem cells. Our recent studies using myeloid leukemic cell lines suggested that induction of differentiation along the myelomonocytic pathway in the presence of differentiating agents renders cells susceptible to productive infection with monocytotropic HIV-1 strains. These studies suggest that earlier precursors, while infectable, may not be appropriate hosts for extensive virus production. In the present report we addressed this question with respect to the regulation of HIV-1 production in normal hematopoietic stem cells and their progeny. We used a CD34+/CD4- BM cell population and examined the kinetics of HIV-1 infection in stem cells. We also assessed the relationship between differentiation and proliferation with respect to productive HIV-1 infection in the progeny of hematopoietic stem cells. CD34+/CD4- BM cells could be infected with the HIV-1m, monocytotropic strain and virus production was enhanced in the presence of IL-3, GM-CSF, and M-CSF. All three hematopoietic growth factors induced macrophage differentiation. However, G-CSF induced neutrophilic differentiation but did not enhance virus production.

The laboratory-maintained HTLV-IIIg strain, which has little tropism for monocytes and macrophages, did not infect CD34+/CD4- BM cells. However, the monocytotropic HIV-1m,F strain consistently infected these cells suggesting that monocytotropism may be important in HIV-1 infection in hematopoietic stem cells. A recent report indicates that HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 that do not include the CD4 binding domain. Our data also suggest that some monocytotropic HIV-1 strains have tropism for hematopoietic stem cells as well as mononuclear phagocytes.

When monocytes were cultured in the presence of

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**Fig 4.** Kinetic analysis of HIV-1 production in CD34+/CD4- BM cells. Sorted CD34+/CD4- BM cells were infected with HIV-1m,F at an MOI = 1 and cultured in the presence of each CSF. The growth factors were used at saturating concentrations: 250 pmol/L GM-CSF, 2.5 ng/mL G-CSF, 1 nmol/L M-CSF, 1 nmol/L IL-3, 1 nmol/L IL-6, and 0.01% bovine serum albumin (BSA) as a control. P24 accumulated production from days 15 to 21 after infection (A) and 3H-thymidine incorporation on day 14 after infection were assayed (B). The experiment was repeated three times and one representative experiment is presented.

**Fig 5.** Effect of hematopoietic growth factors on HIV-1 production and 3H-thymidine incorporation in CD34+/CD4- BM cells. The cells were infected with HIV-1m,F at an MOI = 1 and cultured in the presence of each CSF. The growth factors were used at saturating concentrations: 250 pmol/L GM-CSF, 2.5 ng/mL G-CSF, 1 nmol/L M-CSF, 1 nmol/L IL-3, 1 nmol/L IL-6, and 0.01% bovine serum albumin (BSA) as a control. P24 accumulated production from days 15 to 21 after infection (A) and 3H-thymidine incorporation on day 14 after infection were assayed (B). The experiment was repeated three times and one representative experiment is presented.
with HIV-1 and the amount of virus produced was related to the observations of Griffin et al who showed that HIV-1 in the myeloid lineage are differentiated macrophages more rapidly than freshly sorted cells. This result is consistent with the observations of Griffin et al who showed that HIV-1 expression increased during monocytic differentiation. They postulated that virus production related to an increase in expression of the transcription factor NF-κB. Our results indicate that the most suitable host cells for productive infection by HIV-1 are peripheral blood monocytes as well as hematopoietic stem cells, and circulating blood monocytes as well as hematopoietic stem cells are infected only in patients harboring appropriate strains of HIV-1 and even in these cases the number of hematopoietic stem cells infected may be small.

We have found that the macrophage-active CSFs (GM-CSF, IL-3, and M-CSF) enhanced HIV-1 production in progeny of CD34+/CD4+–BM cells, while G-CSF had no effect on virus production. GM-CSF has been useful in improving hematopoiesis in patients with HIV-1 infection; however, there is a concern that GM-CSF may enhance virus production from macrophages and now, possibly, from BM progenitors. While previous clinical studies did not document increases in serum p24 with GM-CSF therapy, recent reports suggested that GM-CSF alone may have stimulated virus production but combination therapy with AZT may have clinical benefit in HIV-1 infection in vivo.

G-CSF appears to stimulate neutrophil production without affecting HIV production from BM or mature macrophages.

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Table 1. Relationship Between Differentiation and HIV-1 Production in CD34+ BM Cells

<table>
<thead>
<tr>
<th></th>
<th>Fresh CD34+ Cells</th>
<th>Differentiated Cells (preincubation, 7 d)</th>
<th>Differentiated Cells (preincubation, 14 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of macrophage at time of HIV-1 infection</td>
<td>2.5 x 10⁴</td>
<td>2.5 x 10⁴</td>
<td>2.5 x 10⁴</td>
</tr>
<tr>
<td>P24 release per 10⁴ cells (ng)</td>
<td>0.76</td>
<td>1.50</td>
<td>29.16</td>
</tr>
<tr>
<td>The number of cells on day 14 after infection</td>
<td>0.5</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>P24 release per 10⁴ cells (ng)</td>
<td>15.03</td>
<td>18.79</td>
<td>72.88</td>
</tr>
<tr>
<td>Ratio of HIV-1 production</td>
<td>1.24</td>
<td>1.24</td>
<td>47.9</td>
</tr>
<tr>
<td>P24 release per 10⁴ cells from days 36 to 42</td>
<td>79.74</td>
<td>72.76</td>
<td>63.32</td>
</tr>
</tbody>
</table>

Cells were cultured for either 7 or 14 days in the presence of 250 pmol/L GM-CSF and 1 nmol/L IL-3 then exposed to HIV-1, and (MOI = 1).

GM-CSF, IL-3, or M-CSF, the cells were readily infected with HIV-1 and the amount of virus produced was related to [H]-thymidine incorporation. Similarly, virus production in the CD34+ cell population coincided with macrophage proliferation. Differentiated macrophages from CD34+ cells were productively infected more rapidly than freshly sorted cells. This result is consistent with the observations of Griffin et al who showed that HIV-1 expression increased during monocytic differentiation. They postulated that virus production related to an increase in expression of the transcription factor NF-κB. Our results indicate that the most suitable host cells for productive infection by HIV-1 are peripheral blood monocytes as well as hematopoietic stem cells, and circulating blood monocytes as well as hematopoietic stem cells are infected only in patients harboring appropriate strains of HIV-1 and even in these cases the number of hematopoietic stem cells infected may be small.

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G-CSF appears to stimulate neutrophil production without affecting HIV production from BM or mature macrophages.


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Macrophage-active colony-stimulating factors enhance human immunodeficiency virus type 1 infection in bone marrow stem cells

K Kitano, CN Abboud, DH Ryan, SG Quan, GC Baldwin and DW Golde