Hematologic abnormalities are often seen in patients infected with human immunodeficiency virus (HIV). The effect of HIV infection of bone marrow stroma on support of uninfected CD34 progenitor cells in long-term bone marrow culture (LTBMC) was investigated. Results show that HIV-infected bone marrow stroma was unable to adequately support CD34 progenitor cells in vitro. Zidovudine or didanosine was added to cultures in an attempt to reverse the suppressive effects exerted by HIV and to determine whether such suppression was mediated by transfer of HIV infection to progenitor cells. Didanosine failed to reduce the suppressive effects of HIV, whereas zidovudine compounded the observed suppression. HIV infection of bone marrow stroma, while reducing the production of nonadherent cells, did not increase apoptosis and cell death in such cells. In contrast, zidovudine enhanced apoptosis and cell death in nonadherent cells produced by both HIV-infected and control LTBMC.

Hematologic disorders, such as anemia, thrombocytopenia, and other cytopenias, are common in patients infected with human immunodeficiency virus (HIV) and are most frequent in patients with advanced stages of disease [1, 2]. Several mechanisms have been proposed to account for cytopenias, including direct infection of bone marrow progenitor cells [3] and impairment of stromal function [4–9]. In addition, drug therapies used in the treatment of HIV and opportunistic infections may lead to bone marrow suppression.

Bone marrow consists of two closely interacting groups of cells: stromal cells (fibroblasts, adipocytes, endothelial cells, and macrophages) and hematopoietic cells (progenitor and mature cell types) [10, 11]. Growth and differentiation of hematopoietic cells are dependent on their close association with stromal cells, which produce regulatory cytokines necessary to control hemopoiesis [12]. It has been reported that cell types in both stromal and hematopoietic cell compartments are susceptible to infection with HIV in vitro and in vivo.

Previous studies from this and other laboratories have demonstrated macrophages to be the major target cell for HIV infection in bone marrow stroma [13, 14]. There remains much controversy surrounding the possible infection of progenitor cells, with some workers reporting inability to infect progenitor cells in vitro [15, 16] and others demonstrating infection of differentiated progenitor cells [17, 18]. Infection of progenitor cells has been reported in vivo, but only in a subset of seropositive persons [19]. It is therefore unclear whether suppression of bone marrow function is due to direct effects of HIV infection of bone marrow stromal macrophages or whether it is a result of transfer of HIV from infected stroma to circulating progenitors. Bone marrow macrophages are pivotal in support of hemopoiesis by bone marrow stroma; thus, modulation of their function by HIV infection is highly likely to affect hemopoiesis. Furthermore, release of HIV proteins from such cells (gp120, Tat, Nef) may have direct cytotoxic effects on progenitor cells, leading to apoptosis and cell death [4–6].

This study investigated the effect of HIV infection of bone marrow stroma on its ability to support uninfected CD34 progenitor cells. We also determined whether any effects could be countered by use of antiretroviral agents, namely zidovudine or didanosine. Confluent irradiated stroma infected with HIVBa-L was recharged with CD34 cells with and without zidovudine or didanosine. The clonogenic capacity of the progenitor cells and their apoptotic state were assessed weekly by use of semisolid methylcellulose cultures and flow cytometry, respectively.

Materials and Methods

Normal bone marrow. Bone marrow was collected from normal donors by iliac crest aspiration. Bone marrow mononuclear cells (BMMC) were separated by ficoll-hypaque (Pharmacia Biotech, St. Albans, UK) density gradient centrifugation and washed twice in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Paisley, UK).

Long-term bone marrow cultures (LTBMC). BMMC were cultured in 25-cm² tissue culture flasks in IMDM (340 mOsm/kg) supplemented with 10% fetal calf serum (FCS; Sigma Biosciences,
Poole, UK), 10% horse serum (Sigma), 100 IU/mL penicillin-streptomycin (Sigma), 100 IU/mL glutamine (Life Technologies), and 10 \textsuperscript{-4} M hydrocortisone sodium succinate (Sigma), at a concentration of 10\textsuperscript{5} cells/flask in a volume of 10 mL. The flasks were incubated at 37°C in humidified air with 5% CO\textsubscript{2}. Cultures were fed weekly by semi-depopulation of the nonadherent cells and replacement with supplemented IMDM. Cultures were maintained for \textasciitilde4 weeks until stroma reached confluen.

**Elimination of progenitor cells.** At confluence, LTBCM were x-ray–irradiated with a single dose of 1000 rad to eliminate surviving progenitor cells. This was shown to have no effect on HIV infection of LTBCM (data not shown).

**Infection of stroma.** LTBCM were infected with HIV-1 Ba\textsubscript{a-L} (Medical Research Council–AIDS-Directed Programme, South Mimms, UK) using a modification of a method previously described [14]. Briefly, 1.5 mL of cell-free virus stock supernatant, or mock supernatant, was added to each flask and incubated for 2 h, fresh IMDM was added, and the cultures were incubated overnight at 37°C. The stroma was then washed three times with PBS, fresh supplemented medium was added, and the cultures were maintained for \textasciitilde10–14 days.

**Staining and purification of CD34 progenitor cells.** BMMC were stained for CD34 by incubating with the following: human gamma globulins (30 \mu g/10\textsuperscript{6} BMMC; Sigma) for 10 min at 4°C; anti-CD34 antibody (10 \mu L/10\textsuperscript{6} BMMC; QBEND10; Immunotech, Marseilles, France) for 30 min at 4°C, followed by two washes in PBS with 1% FCS; and goat anti-mouse IgG conjugated to phycoerythrin (6 \mu L/10\textsuperscript{6} BMMC; DAKO, High Wycombe, UK) for 30 min at 4°C, followed by two washes in PBS with 1% FCS. The cells were then resuspended in PBS at 10\textsuperscript{6}/mL and kept at 4°C. A negative control was set up concomitantly by use of a nonspecific mouse IgG.

CD34 cells were purified with a cell sorter (FACStar Plus; Becton Dickinson, Oxford, UK). The whole nucleated cell population (excluding red cells and cell debris) was analyzed by use of a scattergram of right-angle light scatter against fluorescence. Regions were drawn defining a population with low right-angle scatter and high fluorescence. This population was then sorted in enrich mode at 5000–6000 cells/s and subsequently in normal-R mode at 200–500 cells/s. Cells were collected into IMDM supplemented with 10% FCS. The frequency of CD34 cells was assessed both before and after sorting, and the purity of the sorted population was then calculated.

**Recharge of CD34 cells.** Purified CD34 cells were added to HIV-infected and control LTBCM (5 \times 10\textsuperscript{5}/flask) in the presence and absence of zidovudine (10\textsuperscript{-5} M; Glaxo-Wellcome UK, Stevenage, UK) or didanosine (10\textsuperscript{-5} M; Bristol-Myers Squibb, Hounslow, UK) between days 10 and 14 after infection.

**Committed bone marrow progenitor assay.** Recharged LTBCM were fed by semi-depopulation weekly, and fresh medium containing either zidovudine or didanosine was added. The nonadherent cells were counted, and 5 \times 10\textsuperscript{5} cells were added to 3 mL of IMDM supplemented with 30% FCS, 1% deionized bovine serum albumin (Sigma), 10\textsuperscript{-4} M mercaptopoethanol, and 0.9% methylcellulose (Stem Cell Technologies, Terry Fox Laboratories, Vancouver, Canada). The growth medium was supplemented with 2 U/mL erythropoietin (EPREX; Cilag, High Wycombe, UK), 5 ng/mL granulocyte-macrophage colony-stimulating factor (1.39 \times 10\textsuperscript{3} U/mg; Novartis Pharmaceuticals, Camberley, UK), and 50 ng/mL interleukin-3 (4.9 \times 10\textsuperscript{6} U/mg; Novartis Pharmaceuticals). Cell-medium suspension (1 mL) was plated into each of two petri dishes and incubated at 37°C in humidified air with 5% CO\textsubscript{2}. After 14 days of incubation, the following colony types were scored: granulocyte-macrophage colony-forming units (CFU-GM), erythroblast–burst–forming units (BFU-E), and mixed colonies (CFU-GEM).

**7-amino actinomycin D (7AAD) staining.** Remaining nonadherent cells taken from LTBCM each week were stained with 7AAD, a fluorescent DNA-binding dye that defines live, apoptotic, and dead populations by flow cytometry [20]. Nonadherent cells (<1 \times 10\textsuperscript{6}) were washed twice in PBS supplemented with 1% FCS and 0.05% Na azide, resuspended in 900 \mu L of PBS and 100 \mu L of 7AAD (200 \mu g/mL; Calbiochem-Novabiochem, Nottingham, UK), and incubated for 20 min at 4°C, protected from light. After centrifugation, the supernatant was removed and the cells were fixed in 500 \mu L of paraformaldehyde (2%). Unstained fixed cells were used as controls.

Samples were analyzed by FACScan (Becton Dickinson) within 30 min of fixing to avoid leaching of 7AAD. Data on \textasciitilde50,000 cells were acquired and analyzed by creating a scattergram of forward light scatter versus 7AAD fluorescence. Regions were defined live, apoptotic, and dead cell populations, respectively, and the proportion of cells within each region was calculated [20].

**HIV-1 p24 core antigen analysis.** Supernatants were collected between days 10 and 14 and assayed for HIV replication before recharge of CD34 cells by p24 ELISA (DuPont NEN, Stevenage, UK), as stipulated by the manufacturer.

**Nucleoside analogue titrations.** To assess the toxicity of zidovudine and didanosine, a series of committed progenitor assays was set up incorporating zidovudine or didanosine at concentrations ranging from 10\textsuperscript{-4} M to 10\textsuperscript{-9} M.

**Statistical analysis.** Statistical significance of data was assessed by application of paired t tests of log-transformed data and multiple comparisons correction.

**Results**

**Effects of HIV infection on production of nonadherent cells by LTBCM.** LTBCM were established from healthy volunteers and x-ray–irradiated to eliminate endogenous progenitor cells. These cultures were incubated with HIV-1 Ba\textsubscript{a-L}, or mock supernatant, and maintained in culture for 10–14 days before recharging with purified CD34 cells. We have previously determined that maximal HIV replication is reached 10–14 days after infection; productive HIV infection of LTBCM in this study was confirmed by p24 ELISA (data not shown) [14]. The total number of nonadherent cells in culture supernatants of LTBCM recharged with CD34 cells was determined weekly. Total numbers of nonadherent cells produced by non-HIV-infected LTBCM increased over the 4-week culture period (figure 1). HIV infection of LTBCM produced a profound reduction in the number of nonadherent cells (P < .05–.01), showing a log-fold reduction by week 4.
Addition of zidovudine to control cultures suppressed CFU-GM, although its effects were less profound than those of HIV infection. Addition of zidovudine to HIV-infected LTBMC had no effect on the reduced CFU-GM numbers observed in nontreated HIV-infected LTBMC (figure 4A). Addition of didanosine had no effect on CFU-GM numbers produced from nonadherent cells from either control or HIV-infected cultures (figure 4B).

Committed bone marrow progenitor assays for BFU-E and mixed colonies (CFU-GEM) were also done on control and HIV-infected LTBMC. Production of these erythroid colonies from control cultures was very short-lived and was not present by week 2 of culture. Although HIV infection of LTBMC appeared to impair the ability of nonadherent cells to produce erythroid colonies, data were not statistically significant because of the transient nature of their production from control LTBMC cultures (data not shown).

To assess effects of antinucleoside analogues in current therapeutic use on production of nonadherent cells by HIV-infected and control LTBMC, cultures were recharged with purified CD34 cells in the presence of zidovudine or didanosine. Addition of zidovudine to control LTBMC produced a suppression of total nonadherent cell numbers similar to that seen in HIV-infected cultures (figure 2A). The greatest suppression of nonadherent cell numbers was observed in HIV-infected LTBMC cultures treated with zidovudine, although this was not statistically significant, suggesting a possible synergistic reduction in production of nonadherent cells. In contrast, didanosine, when added to control cultures, did not produce suppression of nonadherent cell counts (figure 2B). Furthermore, addition of didanosine had no effect on the reduction in nonadherent cell counts observed in HIV-infected LTBMC.

To determine the cytotoxicity of zidovudine and didanosine, short-term clonogenic assays were carried out (table 1). These data demonstrated that zidovudine was cytotoxic at therapeutic levels, while didanosine showed little cytotoxicity at levels far higher than in vivo therapeutic tissue levels.

Effects of HIV infection of LTBMC on production of committed bone marrow progenitor cells. Experiments were done to determine the effects of HIV infection of LTBMC on production of committed granulocyte-macrophage progenitor cells. Nonadherent cells harvested from control and HIV-infected LTBMC, recharged with CD34 cells, were assessed for their ability to form CFU-GM. Nonadherent cells from control LTBMC produced 2–4 x 10³ CFU-GM/week over 4 weeks of culture. HIV infection of LTBMC resulted in significant reduction in CFU-GM numbers ($P < .05–.01$) between weeks 2 and 4, which reached up to 90% reduction in CFU-GM by week 4 after infection (figure 3). This represents a decrease in the absolute number of CFU-GM as well as a reduction in the number of CFU-GM/equal number of nonadherent cells plated.

Figure 1. No. of nonadherent cells, sampled from mock-infected (heavy rule) or HIV-infected (narrow rule) long-term bone marrow cultures recharged with CD34 cells, counted weekly. Data are expressed as means of 6 cultures ± SE.

Figure 2. No. of nonadherent cells, in HIV- (dashed rules) or mock-infected (solid rules) long-term bone marrow cultures recharged with CD34 cells in presence ($10^5$ M; narrow rules) or absence (heavy rules) of zidovudine (A) or didanosine (B), counted weekly. Data are means of 3 experiments ± SE.
Table 1. Committed bone marrow progenitor cells (granulocyte-macrophage colony-forming units [CFU-GM] and erythroid burst-forming units [BFU-E] + mixed colonies [CFU-GEM]) among normal uninfected bone marrow mononuclear cells incubated with or without zidovudine or didanosine.

<table>
<thead>
<tr>
<th>Concentration of zidovudine or didanosine (M)</th>
<th>Zidovudine CFU-GM</th>
<th>Zidovudine BFU-E + CFU-GEM</th>
<th>Didanosine CFU-GM</th>
<th>Didanosine BFU-E + CFU-GEM</th>
</tr>
</thead>
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<tr>
<td>Control</td>
<td>94</td>
<td>52</td>
<td>74</td>
<td>44</td>
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<tr>
<td>10^{-9}</td>
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</tr>
<tr>
<td>10^{-8}</td>
<td>74</td>
<td>16</td>
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<tr>
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<tr>
<td>10^{-4}</td>
<td>3</td>
<td>0</td>
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</tbody>
</table>

NOTE. Results are expressed as colony numbers/10^5 bone marrow mononuclear cells plated.

Effects of HIV infection of LTBMC on progenitor cell viability. Release of HIV and HIV proteins (gp120, Tat, Nef) from HIV-infected bone marrow stroma may have direct cytotoxic effects on progenitor cells, leading to apoptosis and cell death [4–6]. Thus, effects of HIV infection of LTBMC on apoptosis and cell death in nonadherent populations of LTBMC recharged with CD34 cells were investigated. Nonadherent cells were harvested from control and HIV-infected LTBMC and analyzed for levels of apoptosis and cell death by flow cytometry after staining with 7AAD [20]. HIV infection of LTBMC was demonstrated to have no effect on the proportion of apoptotic or dead nonadherent cells compared with nonadherent cells from control LTBMC (P = not significant) (figure 5).

Addition of zidovudine to control or HIV-infected LTBMC increased percentages of apoptotic and dead nonadherent cells by 30%–35% (figure 6A) in nonadherent cells from both con-

![Figure 3](image)

Figure 3. Absolute no. of granulocyte-macrophage colony-forming units (CFU-GM) per mock- (heavy rule) or HIV-infected (narrow rule) flask, recharged with CD34 cells, calculated weekly. Data represent means of 6 cultures ± SE.

![Figure 4](image)

Figure 4. Absolute no. of granulocyte-macrophage colony-forming units (CFU-GM) from mock- (solid rules) or HIV-infected (dashed rules) long-term bone marrow cultures, recharged with CD34 cells, with (narrow rules) or without (heavy rules) zidovudine (A) or didanosine (B) (both 10^{-5} M), calculated weekly. Data are expressed as means of 3 cultures ± SE.
To determine whether suppression of LTBM function observed in this study could be reversed by current nucleoside analogues in therapeutic use, experiments were done in the presence of zidovudine or didanosine. Zidovudine alone was shown to suppress production of both nonadherent and committed progenitor cells in control noninfected LTBM. Zidovudine also produced a possible synergistic suppression of nonadherent cells from HIV-infected LTBM. In contrast, didanosine had no suppressive effects on production of nonadherent and committed progenitor cells from control noninfected LTBM and did not reverse suppressed production of nonadherent cells from HIV-infected LTBM. In this study, we are only comparing the difference in suppression between HIV-infected and control and HIV-infected LTBM. In contrast, addition of didanosine to either control or HIV-infected LTBM had no effect on percentage of apoptotic and dead cells in nonadherent populations (figure 6B).

Discussion

This study demonstrates that HIV infection of LTBM leads to suppression of normal bone marrow function. This was demonstrated by a decrease in production of nonadherent and committed progenitor cells from HIV-infected LTBM recharged with noninfected CD34 progenitor cells. Previous studies have investigated the effects of HIV infection on LTBM function. Continual exposure of LTBM to T cell-tropic strains of HIV has been demonstrated to impair production of nonadherent cells and CFU-GM without significant infection of bone marrow macrophages or progenitor cells [21]. Schwartz et al. [22] demonstrated that infection of LTBM with macrophage-tropic isolate HIV-1ADA, producing endogenous virus within such cultures, reduced CFU-GM. In addition, HIV-2 infection of LTBM has been demonstrated to impair CFU-GM and BFU-E production, dependent on the virus inoculum [5, 23]. Data reported in this study confirm and extend these previous reports.

In contrast, Marandin et al. [24] observed no suppression of CFU-GM or BFU-E production with HIV-1 isolates used in this study. This may have been due to differences in experimental design, as those investigators only examined the clonogenic potential of nonadherent cells at a single time point, and LTBM were recharged 4 weeks after infection, compared with 10–14 days in this study.

Figure 5. Live (solid bars) and apoptotic or dead (open bars) cells among nonadherent cells, sampled weekly from mock- or HIV-infected long-term bone marrow cultures, recharged with CD34 cells, stained with 7-amino actinomycin D. Data represent means of 6 cultures ± SE.

Figure 6. Live (solid bars) or apoptotic or dead (open bars) cells among nonadherent cells, sampled weekly from mock- or HIV-infected, recharged long-term bone marrow cultures in presence of zidovudine (ZID; A) or didanosine (ddI; B) (both 10^{-5} M), stained with 7-amino actinomycin D. Data are expressed as means of 3 cultures ± SE.
noninfected cultures and make no interpretation as to the degree of suppression between HIV-infected conditions, such as in the presence or absence of zidovudine or didanosine. 

Inability of didanosine (used at concentrations known to block HIV infection [data not shown]) to reverse suppressed production of nonadherent and committed progenitor cells from HIV-infected LTBM C demonstrated that such suppression was not mediated by productive infection of progenitor cells. However, this does not rule out the possibility that suppressive effects might occur during interaction of HIV with its receptor or coreceptor or during virus-cell fusion. In addition, such data should be interpreted only within time constraints of the described experiments and do not exclude the possibility that HIV infection of such cells might impair their longer-term viability or function. However, these data demonstrate that failure of bone marrow function is mediated via direct HIV infection of bone marrow stroma alone. We and others have previously demonstrated that bone marrow stromal macrophages are the principal target cells of infection in LTBM C [14, 22, 25]. Thus, suppression of bone marrow function is likely to be mediated either by release of HIV proteins, with suppressive function, from infected macrophages or through impairment of macrophage function, pivotal in normal hematopoiesis.

Previous reports have suggested that soluble HIV proteins (gp120, tat, nef) may mediate inhibition of bone marrow function via induction of apoptosis and cell death in progenitor cells [4–6]. Observations from this study demonstrate that HIV infection of LTBM C, while suppressing overall numbers of nonadherent cells, did not increase levels of apoptosis and cell death in such cells. These data suggest that the decreased production of nonadherent cells is not mediated by a global increase in apoptosis and cell death in this population, suggesting that HIV proteins have no effect on the majority of nonadherent cells. However, such suppression could have been mediated by increased death or lack of proliferation in early pluripotent stem cells, which give rise to the nonadherent population. These cells may be adherent and represent a very small percentage of progenitor cells; thus, increased apoptosis or cell death in such a population would not be detected by assays used in this study. Further studies are currently being done to determine whether the suppressed production of nonadherent cells is mediated by HIV proteins or alterations in bone marrow macrophage function. However, observations that HIV infection of bone marrow macrophages leads to morphologic changes, including giant cell formation [14, 22] and changes in cytokine production [26], strongly suggest the latter to be the predominant mechanism of suppression in HIV-infected cultures.

In contrast to HIV infection of LTBM C, zidovudine treatment of either uninfected or HIV-infected LTBM C induced increased levels of apoptosis and cell death in nonadherent cells. Zidovudine has been reported to have profound effects on hematopoiesis in vivo [27, 28]. This study confirms previous observations and strongly suggests that the observed suppressive effects of zidovudine on hematopoiesis are mediated through induction of apoptosis and cell death in progenitor cells. The mechanism of action is different from that observed for HIV infection of LTBM C. Such data suggest that in HIV-infected patients, HIV infection of bone marrow stroma and zidovudine therapy may act synergistically, through different mechanisms, to impair hematopoiesis. This would suggest that zidovudine treatment should be precluded in patients demonstrating hematologic abnormalities, while didanosine would need no such preclusion.

In conclusion, this study demonstrates that HIV infection of LTBM C induces profound suppression of bone marrow function, resulting in reduced production of nonadherent and committed progenitor cells. Such effects may account for hematologic abnormalities seen in HIV-infected patients and, on the in vitro evidence presented in this study, these would be compounded by zidovudine therapy.

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References


