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In Vitro Suppression of Normal Human Bone Marrow Progenitor Cells by Human Immunodeficiency Virus

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Incubation of normal human nonadherent and T-cell-depleted bone marrow cells with HIV_{IIIB} at multiplicities of infection (MOI) ranging from 0.0001:1 to 1:1 reverse transcriptase (RT) units resulted in the dose-dependent suppression of the in vitro growth of erythroid burst-forming unit (BFU-E), granulocyte-macrophage (CFU-GM), and T-lymphocyte (CFU-TL) colonies of progenitor cells. Maximum inhibition of colony formation was observed at a 1:1 ratio of virus to bone marrow cells. At this MOI, BFU-E and CFU-GM colonies were inhibited by 60 to 80%, while CFU-TL colonies were totally suppressed. Inhibition of colony formation was also observed at an MOI of 0.1:1 but not with further log dilutions of the virus. Incubation of the virus with antibody to gp160 resulted in the complete reversal of stem cell suppression and the normalization of colony growth in vitro. For BFU-E and CFU-GM colonies, this reversal was observed with dilutions of antibody up to 1:100 and was no longer observed at titers greater than 1:500. The CFU-TL colony number normalized at titers between 1:10 and 1:50. Human immunodeficiency virus (HIV) also suppressed by 50% the growth of colonies derived from CD34+ stem cell fractions. Infection of CD34+ cells and T-cell-depleted, nonadherent cell fractions was demonstrated by detection with HIV-specific DNA probe following amplification by polymerase chain reaction. The results suggest that HIV can directly infect human bone marrow progenitor cells and affect their ability to proliferate and give rise to colonies in vitro. The results indicate a direct role for the virus in bone marrow suppression and a possible mechanism for the cytopenias observed in patients with AIDS.

The primary defect in patients who have AIDS resulting from infection with the human immunodeficiency virus (HIV) is a decrease in the number of CD4+ peripheral blood lymphocytes (36). However, impaired hemopoiesis is also characteristic of AIDS and AIDS-related complex, with patients presenting with abnormalities in one or more blood cell lineages resulting in neutropenia, anemia, and thrombocytopenia (5, 13, 19, 21, 25, 30). Several studies have demonstrated decreased in vitro growth of bone marrow progenitor cells from seropositive patients (2, 10, 17, 29). Effects of the virus on accessory cells and the ability of the virus to affect the proliferation and differentiation of progenitor cells have been proposed to cause the hemopoietic abnormalities observed in AIDS patients (7, 16, 31). While Folks et al. demonstrated that HIV could infect and replicate in CD34+ progenitor cells (9), the effect of HIV on the growth of these stem cells and their ability to give rise to colonies in vitro was not examined. The present study examined the effect of HIV infection on the in vitro growth of progenitor cells from normal human bone marrow cells depleted of accessory monocytes and T cells and from CD34+-enriched stem cell populations. A direct inhibitory effect of HIV on the proliferation of these progenitor cells is demonstrated.

MATERIALS AND METHODS

Bone marrow cells. Normal bone marrow cells were obtained from the ribs of patients undergoing thoracotomy or from samples collected during autologous transplantation. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. The cells were washed, resuspended in Iscove's modified Dulbecco's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah), and plated onto plastic dishes to remove adherent accessory monocytes. T lymphocytes were subsequently removed by using a panning technique on immunoglobulin G-coated plates after incubation of the cells with an anti-Leu 3A pan-T antibody (Becton Dickinson, Mountain View, Calif.). The nonadherent, T-cell-depleted (NATD) cells were harvested, washed, and resuspended in Iscove's modified Dulbecco's medium. Purified CD34+ stem cells were obtained by positive selection on mouse immunoglobulin G-coated plates by using HPCA-1 antibody (Becton Dickinson). The adherent cells were harvested by vigorous pipetting and then washed and resuspended in Iscove's modified Dulbecco's medium. By fluorescence-activated cell sorter (FACS) analysis, cells in this fraction were highly purified, with more than 95% of cells being positive for the CD34 marker. Esterase staining shows no evidence of contaminating monocytes in this fraction. Both the NATD and the CD34+ cell populations were completely CD4 negative, as determined by FACS analysis.

Virus, bone marrow infection, and culture. The stock of HIV_{IIIB} used in these experiments had a reverse transcriptase (RT) level of 10^6 cpm/ml (10^6 RT units) as determined by reverse transcriptase assay (14). A total of 10^5 selected NATD or CD34+ progenitor cells were incubated overnight with different log dilutions of HIV_{IIIB} so that the ratios of RT units of virus to cells (multiplicities of infection [MOI]) ranged from 0.0001:1 to 1:1. The cells were then plated in 0.85% methylcellulose (Fisher Scientific, Fairlawn, N.J.) in the presence of 25% fetal bovine serum, 1% deion-

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ized bovine serum albumin (fraction V; Sigma Chemical Co.), 10^{-4} M mercaptoethanol, and the appropriate hemopoietic growth factors. Erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells were assayed in the presence of 2 U of recombinant erythropoietin per ml (University of Pittsburgh), 10 U of recombinant granulocyte-macrophage colony-stimulating factor (gift of Steve Clark at Genetics Institute, Boston, Mass.) per ml, and 5% medium conditioned by the B5637 human bladder carcinoma cell line. T-lymphocyte colonies were grown in the presence of 50 U of recombinant interleukin-2 per ml (AIDS Research and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and 5 μg of phytohemagglutinin per ml. Duplicate cultures were incubated at 37°C and 5% CO_2 for 12 to 14 days, after which colonies consisting of greater than 50 cells were scored under an inverted microscope.

Antibody blocking studies were performed by incubating the virus with different dilutions of anti-gp160 (AIDS Research and Reagent Program) for 2 h prior to the addition of the bone marrow cells to the cultures. Normal goat serum samples served as controls in these experiments. Anti-gp160, which reacts with gp120, was used because of its availability when these studies were undertaken. This antiserum has been shown to react with epitopes that are located exclusively in the gp120 portion of the envelope protein and contains no sequences in the gp41 region (24).

Detection of HIV in bone marrow cells. A total of 10^6 CD34+ or NATD cells were incubated for 8 h with 10^6 RT units of HIV_{IIIB}. The cells were then washed extensively, resuspended in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, and cultured at 37°C. Aliquots of 10^5 cells were isolated at various time intervals, washed twice in cold phosphate-buffered saline, and subjected to lysis at 55°C for 60 min in 10 mM Tris containing 0.5% Nonidet P-40 and 200 μg of proteinase K per ml. The lysate was inoculated for 10 min at 95°C and either used directly for polymerase chain reaction (PCR) amplification or frozen at −20°C. For PCR, 25 μl of extract (1 μg of genomic DNA) was amplified by 35 cycles of reaction with *gag*-specific primers SK38 and SK39 (19) for a 200-bp fragment or SK38 and KW-3′ (5′ GGCTGAATGAGCCAAG TAACAAAT 3′, nucleotide sequence [positions 1422 to 1450] of the *gag* gene) (23) for a 400-bp fragment and analyzed by Southern blot analysis (27) with a nick-translated *gag* probe.

RESULTS

HIV effect on in vitro growth of NATD and CD34+ cells. Increasing the ratio of HIV (RT units) to accessory-cell-depleted normal human bone marrow cells in the incubation mixture from 0.0001:1 to 1:1 resulted in a dose-dependent decrease in the numbers of BFU-E, CFU-GM, and T lymphocyte (CFU-TL) colonies (Fig. 1). Colony formation was inhibited by 40 to 60% at an MOI of 0.1:1. Maximum suppression in the range of 61 to 100% was observed at a 1:1 ratio of virus to bone marrow cells. Table 1 shows the results of several separate experiments in which the effect of HIV on progenitor cell growth was examined at an MOI of 1:1. Overall, CFU-GM, BFU-E, and CFU-TL colonies were inhibited by averages of 61, 70, and 100%, respectively. The colonies scored in these cultures were diminished in size but did not appear to contain virus, as determined by PCR (data not shown). Incubation of uninfected NATD cells with purified gp120 did not affect colony formation (data not shown). HIV also affected the ability of purified CD34+ stem cells to give rise to colonies in vitro (Table 1). Exposure of CD34+ stem cells to HIV at an MOI of 1:1 resulted in a mean suppression of 55% in the total number of colonies derived from these cells. Both CFU-GM and BFU-E colonies derived from this enriched stem cell population were similarly decreased in number.

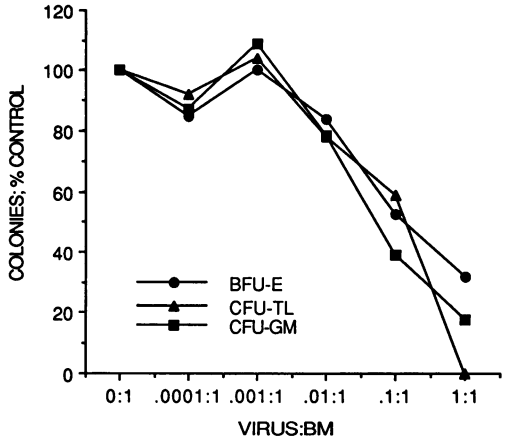

FIG. 1. Effect of HIV_{IIIB} on the in vitro growth of normal human bone marrow-derived progenitor cells. Bone marrow cells were incubated with HIV at different ratios of virus to bone marrow cells (BM). The growth of BFU-E, CFU-GM, and CFU-TL progenitor cells was assessed in methylcellulose cultures, and the results are expressed as percent inhibition of colony formation. The absolute numbers of colonies in control cultures per 10^5 cells plated are as follows: BFU-E, 40; CFU-GM, 120; CFU-TL, 126.

Reversal of suppression by anti-gp160. The effect of antibody to gp160 on HIV's suppression of BFU-E and CFU-GM colonies is shown in Fig. 2. Anti-gp160 reacts with the precursor protein of the gp120 viral surface antigen and cross-reacts with gp120 (24). In the absence of antibody, HIV at an MOI of 1:1 inhibited BFU-E and CFU-GM colonies by 83 and 55%, respectively. A 1:10 dilution of

TABLE 1. Effect of HIV on NATD bone marrow cells and CD34+ cells

Expt and cell group[a]	No. of colonies (% inhibition) for cell type[b]			
	CFU-GM	BFU-E	CFU-TL	CD34+
1				
Control	21	21	ND	47
HIV	2 (95)	9 (57)	ND	22 (53)
2				
Control	30	10	32	24
HIV	13 (57)	3 (70)	0 (100)	9 (63)
3				
Control	61	12	ND	24
HIV	33 (46)	2 (83)	ND	12 (50)
4				
Control	42	ND	64	ND
HIV	22 (48)	ND	0 (100)	ND

[a] Control, Uninfected cells; HIV, cells infected with HIV.

[b] Numbers represent the mean number of colonies in duplicate cultures plated at 4×10^4 cells per well. Numbers in parentheses are the percent inhibition for each data set. CD34+ cells were plated at 4×10^3 cells per well. ND, Not determined.

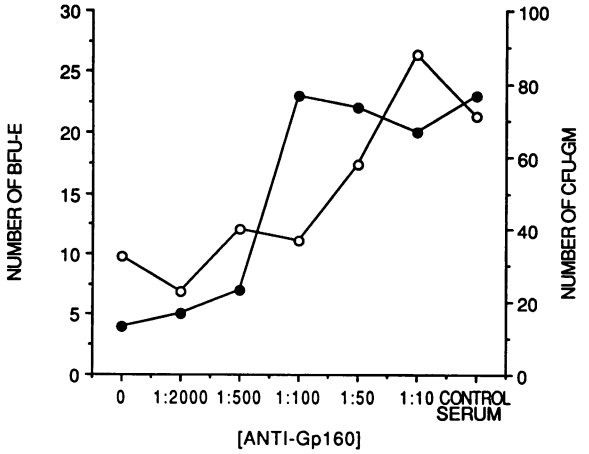

FIG. 2. Effect of antibody gp160 on the HIV-mediated suppression of colony formation by BFU-E (●) and CFU-GM (○) progenitor cells. Virus was incubated with different dilutions of antibody for 2 h prior to the addition of the bone marrow cells. The absolute numbers of colonies in control cultures per 10^5 cells plated are as follows: BFU-E, 34.5; CFU-GM, 97.5.

anti-gp160, which has been shown to cross-react with gp120, completely reversed this inhibitory effect of the virus. A gradual increase in the HIV-mediated suppression of colony formation was again observed as the antibody was diluted to 1:2,000. The suppression of CFU-TL colonies by HIV at a 1:1 ratio was also completely reversed at antibody titers between 1:10 and 1:50 (data not shown).

Evidence for infection of bone marrow cells and CD34+ stem cells. Two days after infection with HIV_{IIIB}, CD34+ or NATD mononuclear cells were harvested, lysed, and subjected to PCR and hybridization as described in Materials and Methods. Figure 3 shows an autoradiograph from a Southern blot of the HIV_{IIIB}-specific *gag* fragments amplified by PCR. With the *gag*-specific primers SK38 and KW-3′, PCR produced a 400-bp *gag*-specific fragment in cells infected with HIV (Fig. 3, lanes 2 and 4). Cells not infected with HIV do not show this band (lanes 1 and 3).

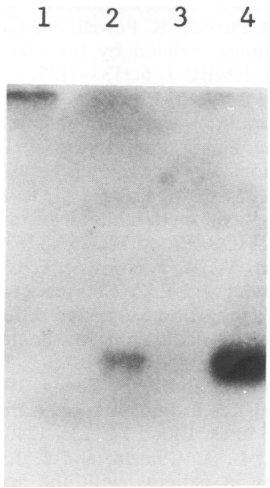

FIG. 3. Southern blot of DNA PCR amplified with *gag*-specific primers. NATD bone marrow cells and CD34+ cells were infected with HIV_{IIIB} at an MOI of 1 RT unit per cell. At two days postinfection, cells were harvested, lysed, amplified by PCR, and run on a 1% agarose gel. The gel was plotted and probed with ^{32}P-labeled *gag* gene. Autoradiograph shows PCR analysis of a 400-bp fragment of the HIV *gag* gene. Lanes 2 and 4, NATD and CD34+ cells, respectively, infected with HIV_{IIIB}; lanes 1 and 3, uninfected NATD and CD34+ cells, respectively.

DISCUSSION

A decline in peripheral blood cell counts, usually involving one or more cell lineages, is characteristic of patients infected with HIV (5, 13, 18, 21, 25, 30, 36). The reported decrease in the in vitro growth of progenitor cells from the bone marrow and peripheral blood of patients with AIDS and AIDS-related complex suggests an etiology of abnormal hemopoiesis and bone marrow suppression (2, 10, 17, 29). HIV-mediated suppression of the bone marrow may result from the activity of the virus directly on the stem cell precursors or on the accessory cells that have important supportive roles in the proliferation and differentiation of these progenitor cells. The possible role for accessory cells in HIV-mediated bone marrow suppression has been reported in a number of in vitro studies using human bone marrow cells and primate animal models (7, 16, 31). Adherent bone marrow cells infected with HIV show a decreased ability to support hemopoiesis in long-term cultures (31). Immune mediated suppression (7) and production of unique inhibitory molecules from infected accessory cells (6, 15, 16) may also represent important mechanisms for the shutdown in hemopoiesis associated with AIDS. Monocytes which appear to serve as reservoirs for the virus may also have a negative influence on the development of blood cells. Recently, Folks et al. suggested a direct role for the virus in bone marrow suppression by demonstrating that HIV could infect and replicate in CD34+ progenitor cells (9). While the virus appeared to have no cytopathic effect on these stem cells, their ability to function normally and give rise to differentiated progeny was not examined. The present study demonstrates that HIV can directly infect normal human progenitor cells and cause a loss in their ability to give rise to colonies of differentiated progeny in vitro. As determined by PCR, the virus is present in NATD bone marrow cells and CD34+ stem cells within 2 days of infection. While the effect of HIV on CFU-TL progenitor cells has been previously demonstrated (17), the present study shows that HIV also negatively affects the in vitro growth of BFU-E and CFU-GM progenitor cells. The effect of HIV on colony formation in bone marrow cells depleted of accessory T cells and monocytes and in purified CD34+ stem cell fractions supports the notion of a direct effect of the virus on the progenitor cells. A new study demonstrating productive infection of HIV type 1 in either normal nonadherent, T-cell-depleted human bone marrow cells or purified CD34+ cells corroborates our findings in this study (12).

This article, which discusses the effect of HIV on progenitor cell growth, is one of an increasing number of reports providing direct evidence for the role of viruses in bone marrow failure and the onset of cytopenias in variously infected patients. An early report by Young et al. established a causal relationship between parvovirus infection and bone marrow failure in patients with aplastic anemia (32). In their studies, a preferential decrease in the number of early BFU-E cells cultured in vitro was observed after normal human bone marrow cells were exposed to the virus. This suppression resulted from a direct effect of the virus on the progenitor cells and was correlated with the replication of

the virus in the bone marrow cells. Nakao et al. recently suggested that the anemia observed in hemorrhagic fever may be related to an effect of dengue virus on early BFU-E progenitor cells (18). Early studies on cytomegalovirus (8, 11) postulated an accessory-cell-mediated mechanism for bone marrow suppression by showing an effect on the stromal cells of the bone marrow, which have an important role in hemopoiesis (1). Recently, Sing and Ruscetti reported that cytomegalovirus can directly affect the in vitro growth of CFU-GM progenitor cells (26). Studies by Steinberg et al. and Zeldis et al. have examined the potential role of hepatitis C and hepatitis B (HBV) type viruses in bone marrow failure (28, 33–35). Acute-phase sera taken from chimpanzees infected with hepatitis C virus inhibited normal human bone marrow progenitor cells (BFU-E and CFU-GM) in vitro (33). Pre-acute-phase and recovery-phase sera did not affect colony formation. Sera obtained from patients infected with hepatitis C virus also suppressed normal colony formation. Exposure of normal bone marrow cells (34, 35) or leukemic cell lines (28) to HBV DNA+ sera or purified Dane particles resulted in a dose-dependent decrease in the number of BFU-E, CFU-GM, CFU-TL, and pluripotential progenitor cells assayed in vitro. The effect of HBV on colony formation was dependent on the MOI and the length of exposure to the virus. The inhibitory effect of HBV could be reversed by antibody to the HBsAg surface antigen of the virus. No inhibitory effect on stem cells was observed with inactivated HBV or with purified viral antigens. As in the case of HBV, the suppression of progenitor cell growth by HIV was dependent on the ratio of virus to bone marrow cells in the incubation mixture. HIV caused a dose-dependent suppression of CFU-GM, BFU-E, and CFU-TL, with significant inhibition evident at an MOI greater than 0.1:1. The lack of a direct effect of HIV on progenitor cells reported in early studies may be related to an inadequate MOI resulting from a low titer of virus in the sera. The reversal of the bone marrow suppression by antibody to HIV envelope antigen (which likely results from antibody binding of the virus) and the lack of an effect of purified gp120 on the growth of the progenitor cells further suggest a direct role for the intact virus in mediating bone marrow suppression.

While disturbances in hemopoiesis in patients with AIDS and AIDS-related complex have been documented, the role of HIV as a cause of bone marrow suppression has been difficult to interpret. Secondary infections with cytomegalovirus or HBV or treatment-induced effects (3, 4) on the bone marrow are likely contributing factors to the abnormal hemopoiesis observed in these patients. Indeed, both cytomegalovirus and HBV have been shown to affect the growth of normal human bone marrow-derived progenitor cells in vitro. While the presence of these viruses in patients with AIDS may play a role in HIV-mediated bone marrow suppression, the present study suggests that HIV itself can directly influence proliferation of bone marrow progenitor cells.

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