Microenvironmental Toxicity of Azidothymidine: Partial Sparing With Hemin


Azidothymidine (AZT) is a useful drug in management of AIDS. Nevertheless, its hematologic toxicity such as anemia and neutropenia present further complications to an already compromised hematopoietic state in patients. We studied the effects of AZT on human and murine bone marrow (BM) colony growth as determined by assays of CFU-E, BFU-E, CFU-GM, and fibroblastoid stromal (CFU-Fb) colonies. Cultures were grown in methylcellulose with growth factors and scored after three- to 14-day incubation. In general, murine marrow cultures were more sensitive to AZT as compared with human marrow. Furthermore, interindividual variation in toxicity to AZT was observed between marrow samples; 1 μmol/L AZT inhibited murine CFU-E, BFU-E, and CFU-GM by 98% to 100%, whereas human marrow was inhibited by 52%, 87%, and 65%, respectively. Lower concentrations of AZT (0.1 μmol/L) inhibited murine erythroid colony growth by 85% to 90%, whereas human growth was inhibited by only 39%.

AIDS IS A FATAL DISEASE caused by a human retrovirus-human immunodeficiency virus (HIV), formerly called human T-cell lymphotropic virus type III/lymphadenopathy-associated virus.1-3 The disease is characterized by severe immunodeficiency, life-threatening opportunistic infections, and neoplasia. Azidothymidine (3'-azido 3'-deoxythymidine) (AZT) is a chain terminator of DNA synthesis and a potent inhibitor of retroviral replication which suppresses the in vitro infectivity and cytopathic effect of HIV.4

Clinical studies show a significant decrease in the incidence of death and opportunistic infections in the AZT-treated patients. This difference is clearly noticeable in AIDS-related complex (ARC). The absolute number of CD4 T lymphocytes also increases during the course of therapy. Therefore, this drug is presently the most useful agent in treatment of AIDS and ARC. However, several cases have demonstrated that AZT administration may be associated with a dose-dependent suppression of BM, resulting in macrocytic anemia and neutropenia.5-7 The mechanisms of AZT-induced anemia and neutropenia are not clearly understood and such cytotoxic effects must be overcome to help maintain adequate hematopoiesis during therapy.8 The significance and mechanisms of this antiviral activity to the observed depression of hematopoiesis is not clear. Certainly, the maintenance of heme synthesis and/or degradation may play important roles in sustaining an adequate level of viable hematopoietic cells.9 Heme is required for cell division and maturation of erythroblasts.10-13 and heme can overcome BM suppression induced by various agents in vitro.14-17 Hematopoietic colony assays are useful tools for evaluating effects on BM progenitor cells by agents administered in vivo or in vitro. We therefore studied the effects of AZT on murine and human BM colony growth and attempted to determine if toxicity could be reversed with any agents such as hemin and growth factors.

MATERIALS AND METHODS

Preparation of cells. The effects of AZT on murine and human BM colony growth were determined by assays of CFU-GM, CFU-E, BFU-E, and fibroblastoid stromal colonies (CFU-Fb). Murine BM cells were obtained from femurs and tibias of DBA/2 female mice, washed and resuspended to the desired concentration in Iscove's modified Dulbecco's medium (IMDM). Human BM cells were obtained by aspiration from the posterior iliac crest from normal donors. In all cases, informed consent was obtained. Low-density cells were obtained by separation ofuffy coat cells by Ficoll-Hypaque, and the nonadherent cells were separated by allowing adherent cells to attach to the bottom of Petri dishes. Cell viability was routinely determined by trypan blue dye exclusion, and viability of all cell preparations was >95%.

Hematopoietic colony assays. Details of methylcellulose technique for BM culture were described previously.18 Murine and human BM erythroid colonies were grown in methylcellulose with 0.4 to 1.0 U/mL erythropoietin added (Epo, Toyobo, Osaka, Japan) for CFU-E and 0.6 to 2.0 U/mL Epo added for BFU-E. CFU-GM were grown with 5% to 10% colony-stimulating factor (CSF, GTCM, GIBCO, Grand Island, NY) in methylcellulose culture. CFU-E growth was scored at 2½ to seven days, and BFU-E and CFU-GM were scored at ten to 14 days.18 The methods for CFU-Fb growth were described previously.19 Aggregates of fibroblastoid cells >50 cells were scored as colonies of CFU-Fb at ten to 14 days.19 All the above tests were performed with and without AZT added (Burroughs Wellcome) at varying concentrations of 0.001 to 10 μmol/L with and without hemin at 10 μmol/L. Hemin (Sigma, St Louis) was dissolved in 0.001 N KOH and diluted in phosphate buffer to a stock concentration of 0.1 mol/L (pH 7.4).

References

HIV infection and HIV antigen (HIV-Ag) detection. Quantitation of HIV infection and replication in human peripheral blood lymphocytes (PBLs) was performed according to previously described methods. PBLs were purified on Ficoll-Hypaque gradients from the blood of healthy donors and resuspended at 2 x 10^6 cells/mL in RPMI 1640 medium supplemented with HEPES, l-glutamine, penicillin, streptomycin, fetal calf serum (FCS), interleukin-2 (IL-2) and phytohemagglutinin-P (PHA) and maintained for three days at 37°C, 5% CO2. On day 3, 2.5 x 10^6 PBLs were aliquoted into a 5-mL RPMI medium without PHA in 25-cm² flasks. Cells were infected with amplified HIV from AIDS patients at 3,000 to 6,000 pg/mL final concentration. Hemin was added to a final concentration of 1 μmol/L, AZT to a concentration of 1 μmol/L, or a combination of 1 μmol/L hemin and 1 μmol/L AZT was added. The cultures were maintained at 37°C with a replacement of 50% of the media after three days containing hemin, AZT, or a combination of hemin and AZT, and the cultures were maintained for four more days. Aliquots were frozen at -70°C with an equal volume of FCS. Samples at day 0 and at the end of the incubation period (end of the experiments) were assayed for HIV-Ag in a solid-phase sandwich-type enzyme assay as described previously with the Abbott antigen detection system to determine picograms per milliliter. This system is used to provide the enzyme label. O-Phenyline diamine is added, and the absorbance is read on a spectrophotometer. Triton X-100 (Sigma) is added to specimens to enhance the sensitivity of the assay. Goat antibody to rabbit IgG conjugated with peroxidase is used to provide the enzyme label. Statistical analysis. Levels of significance for comparisons were determined using t distribution and Wilcoxon signed-rank test. Results are expressed as the mean ± SEM of three to six determinations.

RESULTS

BM hematopoietic colonies were grown in methylcellulose cultures containing Epo or CSF to determine the effects of varying concentrations of AZT on erythroid (CFU-E, BFU-E), CFU-GM, and CFU-Fb colony growth. AZT (10.0 to 0.01 μmol/L) was toxic to murine BM hematopoietic colony growth in all samples tested. Each test was performed in triplicate, and results are the average of six separate determinations. Table 1 shows the effect of various concentrations of AZT on murine BM CFU-E growth. Control CFU-E growth with Epo alone generated 180 ± 12 colonies/2 x 10^5 cells. AZT 1 μmol/L was completely inhibitory to growth, whereas AZT 0.01 μmol/L generated 90 ± 10 colonies, representing 50% inhibition as compared with control growth. The effects of varying concentrations of AZT on the different hematopoietic lineages are shown in Table 2. Results are represented as percentage of control growth; actual colony counts for controls are also shown. With AZT at a concentration of 1 μmol/L complete inhibition of CFU-E, BFU-E, and CFU-GM and 75% inhibition of CFU-Fb growth occurred. At a lower concentration of AZT (0.1 μmol/L), CFU-E and BFU-E colony growth was inhibited by 79% to 90%; the same concentration of AZT inhibited CFU-GM growth by 64% to 71%, but with a moderate inhibition on CFU-Fb. In addition, 0.01 μmol/L AZT inhibited CFU-E growth by 43% to 57%, BFU-E by 64% to 72%, CFU-GM by 38% to 56%, and CFU-Fb by 3% to 27%. These results indicate that AZT has a more toxic effect on CFU-E and BFU-E than on either CFU-GM or CFU-Fb. Addition of 10 μmol/L hemin showed significant reductions in the inhibitory effect for
most cultures with the greatest effect occurring in erythroid and fibroblast cultures (complete reversal of inhibition). Figure 1 shows the effect of hemin on murine colony growth with AZT added. Hemin consistently demonstrated partial to complete reversal of the inhibitory effect of AZT.

In the following series of experiments, AZT was tested for its toxic effect on human BM colony growth. Table 3 shows the effect of AZT 1 μmol/L on erythroid colony (CFU-E, BFU-E) formation. The control with Epo alone generated 158 ± 12 CFU-E/2 × 10⁵ cells, whereas with 1 μmol/L AZT only 76 ± 16 CFU-E were generated, representing 48% of control growth. Similarly, 1.4 ± 1.4 BFU-E were generated/2 × 10⁵ cells with AZT as compared with 94 ± 4.3 BFU-E generated without the drug, representing 87% inhibition of control growth. These inhibitory effects were also obtained with six different healthy normal human BM specimens; data are percentage of control determined from all average values from all specimens.

Because hemin overcame some of the toxic effects of AZT on murine BM hematopoietic growth, we wished to investigate the effects of hemin on human BM clonogenic growth with inhibitory levels of AZT added. As shown in Table 4, hemin reversed the inhibition of CFU-E growth by 0.1 μmol/L AZT to that of control levels (61% ± 8% to 179% ± 26% control), whereas inhibition by 10% CSF and AZT was not reversible (data not shown). In contrast to complete recovery of CFU-E, only partial recovery of BFU-E occurred. As shown in Table 4, only 48% of control BFU-E growth was achieved with the addition of 0.1 μmol/L AZT, whereas 67% of control growth was obtained when hemin was included in culture. AZT has a greater cytotoxic effect on the early BFU-E progenitors as contrasted with the more mature CFU-E progenitors. AZT, at a concentration of 0.1 to 1.0 μmol/L, inhibited human marrow CFU-GM culture growth by 27% and 64%, respectively. Incubation of CFU-GM cultures with hemin resulted in almost complete recovery of AZT (0.1 μmol/L) inhibition of CFU-GM (91% of the control value). However, at a higher concentration of AZT (1.0 μmol/L), partial recovery was observed. Interindividual variations in the sensitivity to AZT were observed among BMs. Analysis of several different BM samples for their sensitivity to AZT suppression yielded variable results. For example, CFU-E growth for six different marrows exposed to 0.1 μmol/L AZT ranged from 46% to 84% of control growth. Inhibition of CFU-GM growth ranged from 43% to 77% of control. Figure 2 summarizes the reversal of AZT-induced inhibition by hemin. Inhibition of human CFU-E growth was completely overcome with hemin, whereas CFU-GM

| Table 3. Effect of AZT on Human BM Erythroid Colony Growth |
|-----------------|-----------------|-----------------|
|                 | CFU-E           | BFU-E           |
| Condition       | No. of Colonies/2 × 10⁵ Cells | No. of Colonies/2 × 10⁵ Cells |
| Control         | 158 ± 12 (100)  | 94 ± 4.3 (100)  |
| AZT (1 μmol/L)  | 76 ± 16 (48)†  | 12.0 ± 1.4 (13.0)‡ |

*Mean ± SEM, n = six determinations (plates). Similar results were obtained in three additional marrows.
†P < .01.
‡P < .001.

| Table 4. Effect of AZT on Human BM Colony Growth With and Without 10 μmol/L Hemin |
|-----------------|-----------------|-----------------|-----------------|
|                 | CFU-E           | BFU-E           | CFU-GM          |
| AZT (μmol/L)    | − Hemin         | + Hemin         | − Hemin         | + Hemin         |
| 0               | 100             | 120 ± 8         | 100             | 124 ± 7         | 100             | 113 ± 9         |
| 0.1             | 61 ± 8          | 119 ± 26†       | 48 ± 2          | 67 ± 2§         | 43 ± 4          | 91 ± 11‡        |
| 1.0             | 52 ± 11         | 112 ± 16‡       | 13 ± 2          | 28 ± 2§         | 36 ± 2          | 66 ± 8§         |

*Cultures were run in triplicate for each individual marrow, and the average values from four normal marrows are shown. Results are the mean ± SEM for 12 determinations.
†P < .001.
‡P < .01.
§P < .05.
GM growth recovered to 66% to 74% of control. A similar but less pronounced effect was noted for BFU-E.

Because thymidine may overcome some AZT toxicity, the next series of experiments was conducted to evaluate whether small molecules such as thymidine may be contaminants in the hemin preparation, because a contaminant may possibly counteract or mimic the hemin effect on AZT-induced inhibition of erythropoiesis. Therefore, we tested several different compounds; results summarized in Table 5 indicate that thymidine (0.1 to 1 μmol/L), thymidine triphosphate (TTP) (0.1 μmol/L), and hemin dialysate have no significant effect on CFU-E growth. These data clearly show that hemin is the major molecule responsible for overcoming AZT-induced inhibition of CFU-E growth.

In another series of experiments, HIV infection and replication in human lymphocyte culture with added hemin and AZT were tested to evaluate whether hemin lowers the antiviral effect of AZT. Results shown in Table 6 indicate that hemin does not lower the antiviral effect of AZT and may even potentiate its action. Hemin in combination with AZT did not reduce the antiviral effect of AZT (72% inhibition) in experiment I and actually enhanced inhibition in experiment II (53% v 33%).

Because hemin overcomes the cytotoxicity of AZT on BFU-E and CFU-GM, hem may stimulate phosphokinase enzyme(s) and enhance metabolism of AZT and lower its action as antiviral agents. Therefore, HIV infection and replication in human lymphocyte culture with added hemin and AZT were tested; results are shown in Table 6. AZT at 1 μmol/L inhibited HIV-Ag content in human lymphocyte culture by 72% and 33% (experiments I and II), respectively. Addition of hemin at a concentration of 1 μmol/L in combination with AZT does not decrease HIV-Ag content. We concluded from these two experiments that hemin does not lower the antiviral action of AZT in lymphocyte culture infected with HIV in vitro.

**DISCUSSION**

The major toxic effect of AZT is on BM, usually causing anemia after the first 6 weeks of therapy. Although the anemia is macrocytic, there is no evidence of disorders of vitamin B12 or folic acid metabolism. The anemia improves in most patients after a reduction in AZT administration.

**Table 5. Effects of Combinations of Hemin and Nucleotide Agents on 0.1 μmol/L AZT-Induced CFU-E Toxicity**

<table>
<thead>
<tr>
<th>Agent</th>
<th>CFU-E Growth (%)</th>
</tr>
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<tbody>
<tr>
<td>Hemin (10 μmol/L)</td>
<td>170</td>
</tr>
<tr>
<td>Thymidine (1 μmol/L)</td>
<td>105</td>
</tr>
<tr>
<td>Hemin vehicle</td>
<td>101</td>
</tr>
<tr>
<td>Thymidine (0.1 μmol/L)</td>
<td>98</td>
</tr>
<tr>
<td>Hemin dialysate (10%)*</td>
<td>96</td>
</tr>
<tr>
<td>TTP (0.1 μmol/L)</td>
<td>95</td>
</tr>
<tr>
<td>AZT + hemin</td>
<td>112</td>
</tr>
<tr>
<td>AZT + thymidine (1 μmol/L)</td>
<td>74</td>
</tr>
</tbody>
</table>

AZT at a concentration of 0.1 μmol/L inhibited CFU-E by 57%. Results are the average of six determinations obtained from two normal marrows. Similar results were obtained in one additional study. Abbreviation: TTP, thymidine triphosphate.

*Hemin was dialyzed overnight in PBS, and the dialysate was tested in CFU-E cultures.

The common finding of one or more cytopenias in patients with AIDS is associated with variable marrow cellularity.21 Similar nonspecific BM changes occur in patients receiving AZT.22

Previous studies in other laboratories have demonstrated in vitro toxicity of AZT to human BM hematopoietic colony growth; the pluripotent stem cells (CFU-GEMM) are the most sensitive.23 In the present study, we showed that murine hematopoietic progenitor cells are much more sensitive to the toxic effects of AZT than is human BM. In this regard, we noted 50% toxicity for murine BM colony growth with 0.01 μmol/L AZT, whereas a similar toxicity for human marrow occurred in a dose range of 0.1 to 0.5 μmol/L. In addition, fibroblastoid stromal cell growth was markedly depressed by similar small concentrations of AZT. Our results indicate that the erythroid series (CFU-E, BFU-E) are relatively more sensitive to the toxic effect of AZT as compared with myeloid precursors and fibroblastoid cells. Experiments in which exogenous hemin was included in BM cultures demonstrated that AZT-induced suppression of colony formation could be overcome and in some instances returned to control levels. However, addition of 0.1 to 1 μmol/L thymidine to the AZT-suppressed CFU-E cultures could not reverse the growth inhibition in a manner similar to that which occurred with hemin. Our results are still consistent with the possibility that higher concentrations (ten- to 100-fold greater than those used in the present study) or prolonged exposure to thymidine may contribute to a protective effect.24 However, hemin alone induces an immediate effect on the heme pathway, independent of thymidine addition, which could account for our results. Even possible contamination of hemin with other small molecules such as thymidine or TTP would have negligible effects as compared with the potent growth-protective effect of hemin.

Hemin induces transformation of 3T3 fibroblasts into adipocytes25 and augments the frequency of primitive erythroid progenitor cells when added to cultures of murine BM cells.11,12,26 Direct administration of hemin to mice resulted in an enhancement of both BFU-E marrow levels and cell cycling within six hours of administration in vivo.27 Inclusion of hemin in BM cultures protected growth of CFU-E that...
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had been exposed to otherwise toxic levels of heavy metals. More recently, administration of heme compounds had beneficial effects on the cytopenias of some patients with myelodysplastic syndrome. The mechanism of action of hemin in promoting growth and differentiation of various cell types remains largely unclear. Hemin may potentiate the action of Epo, as shown in BFU-E and CFU-E growth of sickle cell anemia BM cells. Enhanced expression of specific heme metabolic enzymes may be a mechanism by which hemin induces increases in cell proliferation and/or differentiation. Some of the growth-promoting or mitogenic effects of hemin on erythroid and nonerythroid cells may be related to stimulation of guanylate cyclase. A benefit of hemin is noted in a variety of cellular types and therefore are not restricted to the erythroid lineage. This is not surprising due to the ubiquitous nature of heme itself. Therefore, multiple beneficial effects may be obtained when hemin is administered to HIV-infected BM cells. Although AZT also inhibits DNA polymerase, the DNA polymerase-α present in mammalian cells is ~100-fold less sensitive to inhibition as compared with that of reverse transcriptase. Therefore, the toxicity may be due to different mechanisms such as inhibition of heme synthesis and nucleic acid synthesis and/or replication.

The fibroblasts, endothelial cells, adipocytes, and macrophages are components of the stromal microenvironment. The integrity and interaction of the stromal microenvironment is important in maintaining hematopoiesis, possibly by production of cytokines and growth factors which help provide for maintenance and proliferation of progenitor cells. Some of the growth factors elaborated by stromal cells are CSFs, burst-promoting activity and megakaryocyte colony-stimulating activity. Therefore, the mechanism of AZT toxicity could include interference with components of the stromal microenvironment, resulting indirectly in an increased toxicity to progenitor cells for CFU-E, BFU-E, and CFU-GM.

We suggest that AZT toxicity to hematopoietic cells in vivo may result from a direct or indirect disturbance in the hematopoietic and stromal microenvironment through its inhibition of heme synthesis. Because heme may act synergistically with cytokines and other growth factors, the severity of clinical anemia and/or neutropenia may not be as severe when adequate heme levels are available. Whether such a sparing will occur under in vivo conditions needs to be evaluated. The antiviral effect of AZT in the presence of hemin was also examined in lymphocyte culture of AIDS patients. Preliminary results shown in Table 6 demonstrated that hemin did not antagonize the antiviral effect of AZT in lymphocyte cultures infected with HIV. Indeed, results suggest that in some instances hemin may actually enhance the antiviral effect of AZT. Therefore, whether such a sparing of human BM progenitor cell proliferation and differentiation with added hemin and AZT will occur under in vivo conditions needs to be evaluated.

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