Additive Effect of Erythropoietin and Heme on Murine Hematopoietic Recovery After Azidothymidine Treatment

By Jonathan Harrison, Attallah Kappas, Richard D. Levere, John D. Lutton, Joseph L. Chertkov, Shanlong Jiang, and Nader G. Abraham

The ability of combination treatment with erythropoietin (Epo) and heme to rescue hematopoietic activity in mice from the suppressive effect of azidothymidine (AZT) was determined. Exposure of mice to AZT for 5 weeks produced marked anemia, thrombocytopenia, neutropenia, and weight loss, whereas mice that received Epo and heme for 3 subsequent weeks showed significant alleviation of AZT cytotoxicity. Treatment with Epo (10 U for 5 times/week) stimulated hematopoietic recovery in the AZT-treated animals and reduced the severe anemia and thrombocytopenia by 3 weeks. Administration of a lower Epo dose (1 U Epo) resulted in only a modest retardation of AZT-induced anemia, although, when combined with heme, there was a great improvement in recovery of erythropoiesis. The combination of heme with Epo (10 U) produced the optimum response, resulting in almost normal recovery of bone marrow cellularity as well as recovery of burst-forming units-erythroid (BFU-E) and splenic hematopoietic progenitor content (colony-forming unit-spleen [CFU-S]) by the end of 3 weeks of post-AZT treatment. Treatment with heme alone markedly enhanced the recovery of BFU-E and CFU-S, as well as body weight post-AZT; however, this recovery was not to the extent seen in combination with Epo (10 U). Long-term bone marrow cultures (LTBMCs) established from mice exposed to AZT for 8 weeks showed a marked reduction in cellularity and this was completely alleviated when mice received heme and Epo (10 U) for 3 weeks after 5 weeks of AZT administration. The additive effect of heme and Epo was seen in BFU-E production, as well as in CFU-S production, in LTBMCs. Thus, heme exerts a significant protective effect on hematopoietic progenitors in vivo and may be of potential clinical use in combination with Epo to promote effective erythropoiesis in the setting of AZT therapy.

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AZIDOXYMYDINE (AZT) is the primary therapeutic agent for the management of human immunodeficiency virus (HIV) infection; however, its use is almost always complicated by severe bone marrow (BM) suppression, manifested as anemia and leukopenia. Hematologic toxicity is the major toxicity of AZT, and this is often dose-limiting in the clinical use of AZT. In vitro culture studies have shown that AZT has a suppressive effect on human, as well as murine, BM erythroid (colony-forming unit-erythroid [CFU-E] and burst-forming unit-erythroid [BFU-E]) and myeloid (CFU-granulocyte-macrophage [CFU-GM]) colony formation, and on fibroblastoid cells. An in vitro murine animal model for AZT toxicity has been established and results suggest that AZT has a direct suppressive effect on hematopoietic progenitor cells. In addition, suppression of heme synthesis in vivo by AZT may be one of the mechanisms by which anemia is produced in animals. AZT therapy is associated with an increase in patient survival and multiple strategies have been devised to reduce hematologic toxicity. For example, erythropoietin (Epo) has proven to be effective in partially ameliorating the anemia associated with a variety of disorders, including acquired immunodeficiency syndrome (AIDS). Nevertheless, individual therapeutic agents generally have limited capacity to reverse extreme BM depression, and combination therapies remain promising. One important mechanism by which Epo enhances erythropoiesis may be its direct increase in heme synthesis, however, heme exerts a variety of growth-promoting effects not only on erythropoiesis, but on other hematopoietic lineages. Heme has also been implicated in immune development and function. There is strong evidence that heme can directly influence erythropoiesis. Heme has been shown to directly enhance erythropoiesis and heme-deficient mice have been reported to exhibit suppressed splenic hematopoietic progenitor cell content. Additionally, heme has been shown to indirectly stimulate erythropoiesis through the expression of protooncogenes and through the release of growth factor-like activity. Growth factors have been shown to act both alone and in concert to stimulate hematopoiesis after exposure of the hematopoietic compartment to various anticancer drugs or to toxic compounds such as AZT. Therefore, we examined whether heme alone, or in combination with Epo, would be effective in rescuing hematopoiesis from the toxic properties of AZT. We report that heme has an additive effect with Epo in ameliorating the toxic effects of AZT. Furthermore, our results suggest that treatment with heme and Epo may be of clinical value for correction of the anemia associated with AZT therapy, especially in situations in which Epo alone is found to be ineffective.

MATERIALS AND METHODS

Treatment of animals. Female (C57Bl/6J × DBA/2) F1-specific pathogen-free mice weighing 18 to 20 g and 8 to 12 weeks old were used for all experiments (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions. Body weight and blood indices were determined by routine met-
REVERSAL OF AZT TOXICITY BY Epo AND HEME

...methods. Seven groups of mice, with 8 to 10 mice per group, were studied. These were as follows: (1) normal controls; (2) AZT-treated animals; (3) AZT and heme treatment animals; (4) AZT and Epo (1 U/d); (5) AZT and 10 U Epo; (6) AZT followed by treatments with 1 U Epo plus heme; and (7) AZT followed by treatment with 10 U Epo plus heme. The mice from each group were killed after 8 weeks. Groups of mice exposed to AZT (a gift of Burroughs Wellcome, Research Triangle Park, NC) received the drug in their drinking water at a concentration of 2 mg/mL for 8 weeks. The plasma level of AZT during treatment was maintained at approximately 4 μmol/L. Recombinant human Epo (Toyobo, New York, NY) was administered subcutaneously to specified mice (1 U or 10 U/mouse) 5 times/week for the last 3 weeks of AZT exposure. Heme solution was prepared fresh each time as previously described. A portion of the AZT-exposed mice was treated by heme injections (intraperitoneally) at a dose of 1 μg/g body weight twice weekly during the last 3 weeks of AZT exposure.

Long-term BM culture (LTBMC) and clonal assays. LTBMC was performed using BM cells from controls and treated mice as described previously. All cultures were prepared under sterile conditions. Mice were killed by cervical dislocation, femurs of each mouse were dissected, and the BM was flushed out into a flask (25 cm²; Falcon, Laboratory Disposable Products, North Haledon, NJ) using a sterile syringe and 22-gauge needle with 10 mL of complete culture medium. The media consist of Fischer's medium (GIBCO, Grand Island, NY) enriched with L-glutamine, antibiotics, 10⁻⁶ mol/L hydrocortisone 21-hemisuccinate sodium salt (Sigma Chemical Co, St Louis, MO), and 20% preselected heat-inactivated serum (2:1 horsefet bovine serum, respectively; GIBCO). The culture flasks were tightly capped and kept at 33°C with weekly replacement of 50% of the complete culture medium. During weekly media changes, cells in suspension were counted using a hemacytometer. The cellularity and hematopoietic progenitor content of BM-spleen (CFU-S) and BFU-E of LTBMC were then assayed during the next 10 weeks of culture.

Hematopoietic colony assay. Methylcellulose technique for erythroid (BFU-E) colony assays has been described in detail previously. Erythroid colonies were scored after 12 days of seeding.

CFU-S determination. BM (4 × 10⁶) or LTBMC (2 × 10⁶) cells were injected into mice irradiated with 8.5 Gy from a ¹³⁷Cs-source at a dose rate of 10 Gy/min. Recipient mice were then killed 8 to 9 days after and spleen colonies were counted on spleens fixed with Bouin's solution. All experiments were performed in triplicate. The number of endogenous colonies was less than 0.1 per spleen.

RESULTS

Exposure of mice to AZT for 8 weeks produced marked cachexia, with profound loss of body weight. As seen in Fig 1, body weight in each group did not change significantly during the first 4 weeks. However, there was a gradual decrease in body weight after 4 weeks of AZT exposure, and the decrease became pronounced after 8 weeks of AZT exposure. Administration of Epo alone or in combination with heme was able to significantly attenuate the weight loss (P < .01).

Hematologic parameters in the different treatment groups of animals observed at the end of 8 weeks are presented in Table 1. It can be seen that severe anemia developed in AZT-exposed animals (hematocrit level [Hct], 5.2%) and there was a marked reduction in the platelet count (30,000/μL). In addition, BM cellularity was significantly depressed (1.5 × 10⁵/femur) in animals receiving only AZT, as compared with controls. Treatments with heme, Epo, or combinations of the two agents consistently produced improvements in all the hematologic parameters monitored. In particular, the combination of heme with the higher dose of 10 U Epo produced the greatest improvement. Treatment of AZT-exposed mice with heme plus 10 U Epo resulted in animals having near normal BM cellularity (8.8 ± 0.5 × 10⁵/femur) and Hct levels (44.6%) as compared with controls, despite receiving AZT in the period between 5 and 8 weeks. In addition, platelet numbers were slightly greater than normal control values (260,000/μL ± 200,000/μL). Similar findings on the effect of Epo on platelets and megakaryocytes have been reported.

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<th>Table 1. Hematologic Parameters of AZT-Exposed Mice (8 Weeks) Treated With Epo or Heme Alone or in Combination</th>
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* AZT administered in drinking water (2 mg/mL) for 37 days so that plasma levels equal 4 μmol/L.
† Heme was administered intraperitoneally (1 μg/g) 2 times/week for the last 3 weeks of AZT exposure.
‡ Indices were examined at 8 weeks with 8 mice per group. Differences are statistically significant at P < .01 in AZT-exposed mice receiving heme or Epo or combinations as compared with AZT treatment alone.
§ Epo (1 U or 10 U/mouse) was administered subcutaneously 5 times/week for the last 3 weeks of AZT exposure.
Furthermore, it was evident that AZT exposure produced a notable decrease in Hct levels (15.1%) and cellularity (4.5 \times 10^6 cells/femur) after 4 weeks of exposure. A similar trend was seen for AZT-exposed animals receiving heme (36.3%; 5.8 \times 10^6 cells/femur), 1 U Epo (35.2%; 5.0 \times 10^6 cells/femur), and 10 U Epo (34.3%; 6.2 \times 10^6 cells/femur). Additionally, minor reductions were seen at 4 weeks of AZT treatment but receiving heme plus 1 U Epo (36.2%; 5.9 \times 10^6 cells/femur) and heme plus 10 U Epo (46.9%; 8.9 \times 10^6 cells/femur). Quantitation of the number of CFU-S showed a pattern of response similar to the other hematologic parameters noted above. Exposure of mice to AZT for 8 weeks markedly depressed the CFU-S content (Fig 2). The CFU-S counts obtained from AZT-exposed mice were only 0.3 ± 0.05 \times 10^3 CFU-S/femur after 8 weeks of AZT exposure, whereas the control value was 3.8 ± 0.3 \times 10^3 CFU-S/femur. Pretreatment with 1 U Epo for 3 weeks produced only a modest (but not significant) increase in CFU-S/femur as compared with AZT exposure alone (P < 0.05). In contrast, administration of 10 U Epo resulted in significant hematopoietic progenitor cell recovery from AZT effects, with the group treated with 10 U Epo and heme yielding CFU-S numbers greater than control levels. For example, heme plus 10 U Epo/d treatment elevated the CFU-S content to 5.4 ± 0.4 \times 10^3/femur, whereas heme treatment alone gave rise to a CFU-S content of 3 ± 0.2 \times 10^3/femur (Fig 2).

Erythroid (BFU-E) progenitors in AZT-exposed mice were also severely depressed; however, 1 U Epo or heme treatments alone caused a partial recovery (Fig 3). In contrast, a combination of 1 U Epo and heme or 10 U Epo and heme caused a complete recovery of BFU-E activity, despite prolonged AZT exposure (P > 0.01). Although the levels of BFU-E/femur in 1 U Epo and the heme treatment group were not significantly different from that of the group in which a higher dose of 10 U Epo was administered, the average number of BFU-E/femur was in fact twofold higher in mice treated with a combination of 10 U Epo and heme than in animals treated with 1 U Epo and heme. In Fig 3, it can be seen that the number of BFU-E/femur in 10 U Epo mice was 7.1 ± 2 \times 10^3 as compared with a BFU-E count of 19.8 ± 3 \times 10^3 in mice treated with 10 U Epo plus heme. It is also clear that treatment with heme alone, or Epo alone, improved the BFU-E counts of AZT-exposed animals to near control levels. For example, heme alone resulted in the generation of 4.2 ± 0.4 \times 10^3 BFU-E/femur, and 1 U Epo or 10 U Epo gave 3.2 ± 0.5 and 5.9 ± 0.4 \times 10^3 BFU-E/femur, respectively.

The ability of BM cells from AZT-exposed mice to sustain hematopoiesis in LTBCMC was also examined. The cumulative cell production during 8 weeks in LTBCMC treatment for each group is shown in Fig 4. AZT exposure alone produced a marked reduction in total cellularity during the 8 weeks as compared with controls (9 \times 10^9/culture \times 67 \times 10^4/culture for controls). Administration of heme to AZT-exposed mice did not enhance cell number in LTBCMC to the same degree as for LTBCMC from AZT-exposed mice treated with 10 U Epo. However, a combination of both 10 U Epo and heme to AZT-exposed mice increased LTBCMC BM cellularity more than either agent alone (55 \times 10^9 cells/culture as compared with 21 \times 10^9 cells in heme or 38 \times 10^9 cells in 10 U Epo AZT-exposed mice).

Determinations of BFU-E progenitors in LTBCMC (5 weeks) were also performed, and these results are shown in Fig 5. It is clear that there is a marked reduction in the
number of BFU-E derived from LTBMCs established from AZT-exposed mice, and that treatment of animals with heme plus 10 U Epo produced the most enhanced BFU-E growth response in LTBMC. The number of BFU-E/culture obtained from control animals was 700 ± 51/culture, as contrasted with 2,350 ± 110 BFU-E/culture obtained from the AZT-exposed animals receiving heme plus 10 U Epo (Fig 5). Treatment of AZT-exposed animals with heme or Epo alone produced improvements in BFU-E formation to levels approximately those seen in non-AZT-treated controls (Fig 5).

**DISCUSSION**

The observations made during the present study clearly show that coadministration of Epo and heme to AZT-exposed animals significantly alleviates hematologic toxicity and improves long-term progenitor repopulating capacity. Treatment of AZT-exposed mice with either Epo or heme alone produced improvements in all of the hematologic parameters studied, but to a lesser extent than their combination. The results presented here show that hematologic recovery is not limited to any one lineage, because marked improvements were seen in platelet counts as well as measures of erythropoiesis. Similar findings of a positive effect by Epo on platelets and megakaryocytes have been reported in other experimental settings.20,21 Mice receiving AZT for 5 weeks experienced reductions in hemoglobin levels, Hct levels, and platelet counts, as well as a 65% loss of body weight as compared with controls. After a 3-week period of administration of 10 U of Epo to animals that had previously received AZT, there was a partial restoration of erythropoiesis and improvements in body weight. In contrast, a 3-week rescue with 1 U of Epo alone appeared to have little effect on the recovery of erythropoiesis in AZT-treated mice. The administration of 1 U Epo in combination with heme resulted in a slight improvement in hematopoietic recovery that was not seen with 1 U of Epo alone. Thus, coadministration of heme with Epo at either 1 U or 10 U resulted in enhancement of both erythropoiesis and platelet production.

The beneficial effect of Epo (10 U) on erythropoiesis extending to other hematopoietic cell lineages is not unique to this experiment. It has been shown that Epo enhances megakaryopoiesis in vitro and in vivo.20,21 In addition, a variety of cell lines respond to Epo,22 including early hematopoietic progenitor cells.23 The exact mechanism for the Epo effect on cell lineages other than erythroid is not known.24 However, one of the unique findings presented here is that heme appears to potentiate the effect of Epo in rescuing hematopoietic progenitors from the cytotoxicity of AZT in vivo.

Consistent with the positive effect on erythropoiesis and platelet production by heme, either alone or in combination with Epo, was a greater than sixfold enhancement of CFU-S counts in the marrow within 3 weeks after therapy. Because BFU-E activity was reduced to about 20% of control levels within 5 weeks of initiating AZT treatment, it is unlikely that the proliferation and expansion of surviving BFU-E or CFU-S alone could account for this level of recovery over such a short interval of time. Rather, the enhanced recovery of the BFU-E observed at 3 weeks of rescue by heme and Epo in AZT-treated mice may very well result from accelerated self-renewal and differentiation of a more primitive stem cell subpopulation that had survived the AZT insult. It
is possible that this primitive subpopulation may be expanded through the action of heme, alone or in combination with Epo, either directly or perhaps through accessory cells of the hematopoietic stroma. This suggestion arises from previous observations that heme causes the release of hematopoietic cells from the adherent cell layer (ACL)\(^1\), release of hematopoietic cells from their site of early development and nurture would be expected to be accompanied by a process of maturation. Consequently, heme may induce mobilization of colony-forming cells with high proliferation potential toward the erythroid compartment from the ACL. More recently, heme has been shown to enhance CFU-S in suspension of LTBM.C.\(^1,4\) Others have shown that heme increases early progenitor cells; for example, heme increases pluripotent CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) numbers in cultures.\(^3,15,23\) Thus, at a cellular level, this model suggests that heme plays a role in stimulating an earlier stage of hematopoiesis than Epo alone stimulates and that the joint effects of these two agents are complementary.

The present studies strongly argue that heme, in combination with Epo, not only enhances cellularity of the marrow after a toxic insult, but also mobilizes the stem cell compartment (Table 1).

The observed additive effect between heme and Epo seen in the present experiment may be another example of the effective use of growth factors in combination. Previous studies of the effect of cytokines on human hematopoietic cells have indicated very limited activity when used singularly on hematopoietic progenitors,\(^6\) with the possible exception of megakaryocyte precursors\(^29\) and activated B cells.\(^30\) However, combinations of several growth factors can exert stimulating activity on very primitive human cells,\(^26,28\) including multipotential blast colony-forming cells.\(^31,34\)

Thus, in summary, the evidence of the cooperative interaction between heme and Epo in the present report holds promise as a means to ameliorate the toxicity of AZT in patients. Epo used in combination with heme in the treatment of AZT-suppressed hematopoiesis appears possible. Epo alone may not be able to fully correct the anemia in all HIV patients undergoing AZT therapy. Thus, use of both Epo and heme may prove beneficial for patients in whom anemia is not responding to the usual regimen of Epo alone.

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